

THE ANALYSIS OF ANTIBIOTICS USING
ELECTROSPRAY QUADRUPOLE ION TRAP MASS SPECTROMETRY:
INFRARED MULTIPHOTON DISSOCIATION AND
LIQUID CHROMATOGRAPHY/MASS SPECTROMETRY

By

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TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS	ii
ABSTRACT	vii
CHAPTERS	
1 INTRODUCTION	1
Overview of Dissertation	2
Antibiotics	4
Electrospray Ionization	5
Non-Covalent Associations	7
The Quadrupole Ion Trap Mass Spectrometer	10
History	10
Principles and Theory of Operation	13
Collision-Induced Dissociation	15
Infrared Multiphoton Dissociation	17
High Performance Liquid Chromatography/Electrospray Mass Spectrometry	20
2 IRMPD EXPERIMENTS WITH A CONSTANT PRESSURE OF HELIUM BUFFER GAS USING A PULSED CO ₂ LASER	22
Introduction	22
Experimental	27
IRMPD Instrument and Procedures	27
Electrospray Conditions for the Vancomycin-Ac ₂ KAA Complex	31
Results and Discussion	32
IRMPD of Erythromycin	32
IRMPD of Neomycin	36
IRMPD of Tylosin	38
IRMPD of Vancomycin	40
Analysis of the Vancomycin-Ac ₂ KAA Non-Covalent Complex	40
IRMPD of the Vancomycin-Ac ₂ KAA Non-Covalent Complex	43
Conclusions	45

3	INVESTIGATIONS OF THE COLLISIONAL QUENCHING OF PHOTO-EXCITED IONS DURING THE IRMPD PROCESS	47
	Introduction	47
	Experimental	49
	Results and Discussion	51
	IRMPD Efficiency as a Function of Pressure	51
	Ion Injection Using a Short Pulse of Helium	54
	Scan Function Using a Pulsed-Inlet of Helium for IRMPD Studies	60
	IRMPD of Erythromycin Using a Pulsed-Inlet of Helium	62
	IRMPD of Vancomycin Using a Pulsed-Inlet of Helium	62
	Conclusions	65
4	IRMPD EXPERIMENTS WITH A PULSED INTRODUCTION OF HELIUM BUFFER GAS USING A PULSED CO ₂ LASER	67
	Introduction	67
	Experimental	69
	Results and Discussion	70
	IRMPD Erythromycin Using a Pulsed-Inlet of Helium	70
	Collision-Induced Dissociation Versus IRMPD of Erythromycin	76
	IRMPD of Neomycin Using a Pulsed-Inlet of Helium	78
	Collision-Induced Dissociation Versus IRMPD of Neomycin	83
	IRMPD of Tylosin Using a Pulsed-Inlet of Helium	85
	Collision-Induced Dissociation Versus IRMPD of Tylosin	88
	IRMPD of Polymyxin B ₁ Using a Pulsed-Inlet of Helium	88
	Conclusions	90
5	QUANTITATIVE DETERMINATION OF POLYMYXIN B ₁ IN HUMAN PLASMA WITH LC/MS UTILIZING ELECTROSPRAY IONIZATION COMBINED WITH A QUADRUPOLE ION TRAP	94
	Introduction	94
	Experimental	96
	Materials, Reagents, and Standards	96
	Preparation of Standards	97
	Pretreatment of Plasma Standards	97
	SPE Plasma Sample Preparation for LC/MS Assay	98
	High-Performance Liquid Chromatography/Electrospray Mass Spectrometry	100
	Results and Discussion	103

Characterization of Polymyxin B ₁	103
Method Development for Plasma Extraction.....	111
LC/MS Results for the Recovery of Polymyxin B ₁ in Human Plasma: Calibration Curves for Day 1 and Day2	114
Recoveries of PMB ₁ in Spiked Plasma.....	116
Analysis of Samples.....	116
LC/MS Assay Precision.....	118
LC/MS Assay Accuracy	120
Linearity of Polymyxin B ₁ Peak Areas for LC/MS Assay	121
Reserpine Area Statistics	124
Conclusions.....	127
 6 CONCLUSIONS AND FUTURE WORK	 130
 LIST OF REFERENCES.....	 134
BIOGRAPHICAL SKETCH	143

Abstract of Thesis Presented to the Graduate School
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TRAP MASS SPECTROMETRY: INFRARED MULTIPHOTON DISSOCIATION
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Major Department: Chemistry

The infrared multiphoton dissociation technique combined with electrospray ionization/quadrupole ion trap mass spectrometry has been used for the structural analysis of antibiotics. Currently, the method of choice for ion activation during ion trap experiments is collision-induced dissociation. However, due to several limitations of the collisional activation technique, photodissociation has become an effective alternative for the structural elucidation of several important classes of biomolecules. This dissertation presents the infrared multiphoton dissociation results for several antibiotics using novel techniques developed here at the University of Florida, and also presents the results from an LC/MS assay developed for the quantitative determination of polymyxin B₁ in human plasma.

Previous infrared multiphoton dissociation experiments using a quadrupole ion trap have produced limited photodissociation efficiencies due to the relatively high pressure of helium buffer gas continuously infused into the analyzer region to improve ion trap performance. Experiments have indicated that uncorrected pressures below 2×10^{-5} torr are necessary to avoid collisional deactivation of photoexcited ions. In order to enhance photodissociation results, a dual pulsed introduction of helium was incorporated into the ion trap scan function, allowing for removal of helium buffer gas during laser irradiation.

Next, direct comparisons were made between infrared multiphoton dissociation and collision-induced dissociation for several high molecular weight antibiotics. The results indicated an enhanced ability of the photodissociation technique combined with the ion trap to detail structurally significant low m/z daughter ions when compared to collisional activation. Preliminary results have also indicated the photodissociation process is useful for the MS/MS analysis of non-covalent associations. The well-defined energy deposition of the photodissociation technique offers the ability to preferentially cleave the weaker non-covalent bonds of many adducts formed during the gentle electrospray ionization process.

Finally, the results from an LC/MS assay for the quantitative determination of polymyxin B₁ in human plasma are presented. Using reserpine as an internal standard, plasma samples were examined on two different days. Calibration curves obtained indicated good linearity, but poor precision and accuracy. A detailed explanation of problems encountered is presented, and improvements to the method, including an LC/MS/MS assay, are suggested.

CHAPTER 1 INTRODUCTION

Recently, pharmaceutical companies have resumed research and development of new antibiotics. The realization that bacteria are developing resistance to current antibiotics administered has triggered this renewed interest. Vancomycin, a peptide antibiotic whose efficacy was long thought to be impervious to bacterial mutations, is a case in point. One genus of bacteria, the *Enterococcus* subclass of *Streptococci*, has acquired resistance to vancomycin, and other types of bacteria may soon follow.¹

To aid the bioanalytical mass spectrometrists in drug discovery, new approaches to structural elucidation of antibiotics are necessary. Currently, the technique of choice for ion activation using the quadrupole ion trap mass spectrometer (QITMS) is collision-induced dissociation (CID).² The advantages of CID in the ion trap include the efficient conversion of parent ions to daughter ions, and the high collision cross sections observed.^{2,4} However, several researchers have recently been investigating alternative methods for ion activation in the QITMS, including infrared multiphoton dissociation (IRMPD), or what is frequently called IR photo-induced dissociation (PID). On a novel electrospray/ion trap mass spectrometer built in our laboratory for photodissociation studies,⁵ four classes of compounds have been studied: oligonucleotides, peptides, oligosaccharides, and carbohydrate antibiotics.⁵ Due to the high photon absorption cross

section of the C-O-C linkage at the wavelength of the CO₂ laser (10.6 μ m), IRMPD provides detailed structural information when cleaving the glycosidic bonds of carbohydrates and specifically carbohydrate antibiotics.^{6,7}

This dissertation will first present an alternative method for the activation of antibiotics in the QITMS. Several antibiotics, including erythromycin and vancomycin, will be examined using both continuous wave (cw) and pulsed CO₂ lasers. Strategies for improving the efficiency of the IRMPD process within the ion trap will be highlighted, including the use of a dual pulse inlet of helium buffer gas to reduce the collisional quenching of photoexcited ions. Also, direct comparisons with the more conventional resonant excitation method of CID will be made, and the advantages of the IRMPD process for the structural elucidation of antibiotics will be summarized.

Finally, an LC/MS assay for the peptide antibiotic polymyxin B₁ in human plasma will be discussed. The assay developed provides a sensitive technique necessary for the determination of low levels of polymyxin B₁ for bioavailability and toxicokinetic studies. Electrospray LC/MS is ideally suited for the analysis of polymyxin B₁ in plasma; data will be presented detailing the ability of the ion trap mass spectrometer to monitor both PMB₁ and the internal standard reserpine.

Overview of Dissertation

This introductory chapter begins with a brief description of antibiotics, followed by a discussion of the electrospray ionization (ESI) technique used to generate gas-phase ions for the studies presented in this dissertation. Next, a brief introduction on non-covalent associations will be presented. Also, a brief history of the ion trap will be given,

along with the general operating principles of the Finnigan MAT ion trap mass spectrometer (ITMS). Also, the principles of two ion activation techniques, CID and IRMPD, using the quadrupole ion trap will be examined. Finally, a brief discussion on HPLC combined with electrospray ionization/mass spectrometry will conclude the chapter.

Chapter 2 presents the first photodissociation results with antibiotics using a pulsed CO₂ laser with a constant pressure of helium buffer gas. Several antibiotics were chosen as probes, including erythromycin, neomycin, tylosin, and vancomycin. Also, the non-covalent interaction of vancomycin with the peptide N,N-diacetyl-L-Lysine-D-Alanine-D-Alanine (Ac₂KAA) will be examined using IRMPD. Chapter 3 examines the effect of collisional quenching of photoexcited ions. A scan function is presented utilizing a pulsed-inlet of helium to allow for lower pressures during laser irradiation. IRMPD data using a continuous wave (cw) CO₂ laser is presented.

Using this dual introduction of helium, data is presented in chapter 4 using a pulsed CO₂ laser. Several antibiotics are examined with the IRMPD process, including erythromycin, neomycin, and tylosin. Comparisons with both collision-induced dissociation (CID) and IRMPD using a constant pressure of helium are presented. Finally, chapter 5 presents an LC/MS assay for the quantitative determination of polymyxin B₁ in human plasma utilizing electrospray ionization combined with the quadrupole ion trap. A solid phase extraction (SPE) technique was developed for sample clean up. A basic peptide, reserpine, was chosen as the internal standard for the assay. LC/MS assay linearity, precision, and accuracy are discussed in detail. Chapter 6 summarizes the research reported in this dissertation and presents the future work to be

done in both the areas of IRMPD of antibiotics, and the development of an LC/MS/MS assay for PMB₁ in plasma.

Antibiotics

Antibiotics (“anti-life”) are substances derived from microbes, primarily found in soil, that kill other bacteria. The term has been extended to include synthetic compounds that inhibit microbes.⁸ Penicillin was the first antibiotic discovered, in 1928. Shortly thereafter, antibiotics were seen as wonder drugs able to eliminate many potentially fatal bacterial infections. However, doctors gradually expanded the use of antibiotics to infections that were not life-threatening, and even to infections that antibiotics were ineffective against. Unfortunately, over time bacteria developed the ability to resist antibiotics that once destroyed them. This bacterial resistance has led drug companies to search for newer, more potent antibacterial agents.

New antibiotics are discovered by two methods: screening and selective modification.¹ Screening starts with expeditions sent out to collect soil samples from different locations. Next, the various organisms are isolated from the soil and grown in a “fermentation broth”. This broth is tested for efficacy on various bacteria. Unfortunately, this process is time-consuming and not very cost-effective. Selective modification involves modifying a known antibiotic in order to increase efficacy. The most common modification involves adding side chains to the core antibiotic structure. Another approach, developed recently, uses our knowledge of molecular biology to look for bacterial physiologic processes that can be used as antibiotic targets.¹

Antibiotics are often classified by their action mechanism against bacteria.⁸ There are two groups of antibiotics that alter bacterial cell wall synthesis. The first group

includes the beta-lactams, the penicillins and cephalosporins, which inhibit cross-linking of the cell wall structure and cause increased permeability, leakage, and death. A second group of antibiotics inhibit the second stage of cell wall growth; this group includes cycloserine, bacitracin, and vancomycin. A second class of antibiotics inhibits bacterial protein synthesis. This group includes the aminoglycosides, tetracycline, chloramphenicol, and erythromycin. A third class of antibiotics acts on the bacterial cell membrane causing increased permeability and leakage of cell contents. Drugs in this class include polymyxin B, nystatin, and amphotericin B. Lastly, a class of antibiotics inhibits nucleic acid synthesis. This class includes rifampin and the quinolone group of antibiotics.

Electrospray Ionization

In the past decade, electrospray ionization (ESI) has emerged as a powerful technique for producing gas-phase ions from large and complex biomolecules in solution.^{9,11} The capabilities of electrospray ionization coupled with mass spectrometry include the detection of polar, labile compounds with relative molecular masses approaching the 10^5 range and beyond.⁹ Of particular note are the multiply charged ions characteristic of the electrospray process, which enable the detection of large biomolecules with inexpensive quadrupole-based mass spectrometers. The gentle ionization characteristics of the electrospray method have also allowed researchers to examine non-covalent associations.¹² Also, the ability to combine separation techniques like capillary electrophoresis and microcolumn liquid chromatography with electrospray/mass spectrometry^{13,14} has been a large impetus in the popularity of the ionization method.

Although researchers had examined the electrospray phenomenon earlier, the first use of electrospray as an ionization method was by Dole and co-workers.^{15,16} These researchers were examining the potential of electrospray to produce gas-phase macro-ions. Experimental results were presented for the ionization of the proteins zein (MW $\approx$ 50,000) and lysozyme.^{17,18} Unfortunately, a mass spectrometer was not available to aid the workers in interpreting these early results.

The first successful combination of electrospray with mass spectrometry was reported in 1984 by Yamashita and Fenn,¹⁹ and by Aleksandrov et al.²⁰ Fenn later went on to prove electrospray's capabilities for extensive multiple charging^{21,22} and the mass spectrometry of large biomolecules.^{23,24} Although Fenn was instrumental in the development of ESI/MS, many of the subsequent applications of the new technique took place in the groups of Smith,¹⁰ and Henion.^{12,25} Several excellent reviews are available on the development and applications of electrospray ionization.⁹⁻¹¹

Electrospray ion production is a two-step process: dispersal of highly charged droplets at near-atmospheric pressure, followed by conditions resulting in droplet evaporation.¹⁰ An electrospray is produced by applying a high electric field to a small flow of liquid (generally 1-20 μ L/min) from a stainless steel needle. A potential difference of +3-6 kV is typically applied between the needle and the chamber and surrounding cylindrical electrode located 0.3-2 cm away. By switching the polarity of the needle, negative ions can be produced. The electric field charges the surface of the emerging liquid, dispersing it by repulsive Coulombic forces to form a fine spray of charged droplets. Solvent evaporates from each droplet as the droplets migrate toward a

heated capillary (200°C). Older source designs applied a countercurrent flow of bath gas at a temperature of 300°C.^{10,11} Recent source designs as shown in figure 1-1, apply a coaxial flow of nitrogen (positive ions) or oxygen (negative ions) to aid the desolvation process. Once droplets enter the heated capillary, the rate of solvent evaporation is hastened, decreasing the droplet diameter and increasing the charge density of the surface of each droplet.

Two mechanisms have been developed to explain electrospray ion formation: a cascade of fission processes at the Raleigh limit,²⁶ and direct field evaporation of ions.²⁷ The generally accepted theory for ion formation is one set forth by Iribane and Thomson.^{28,29} They postulated that droplet fission occurs by a cascade of fission processes yielding smaller and smaller droplets until the the radius of curvature of a droplet becomes small enough that the field due to surface charge density is strong enough to desorb ions. Although a debate exists over the exact mechanism of ion formation during the ESI process, electrospray allows any species that carries a net charge in solution to be transferred to the gas phase after evaporation of residual solvent.¹⁰

Non-Covalent Associations

Both matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization when combined with mass spectrometry have enabled the detection of large, labile biomolecules. However, when compared to MALDI, electrospray is a more gentle ionization technique^{10,11} that has become essential for the analysis of a broad spectrum of biologically important compounds. Of particular biological interest are non-covalent associations in solution which can be studied in the gas phase by ESI/MS. The strengths

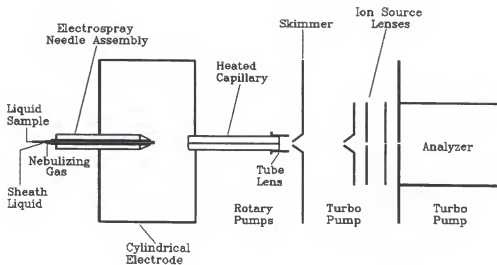


Figure 1-1. Schematic of electrospray source containing a coaxial nebulizing gas and a heated capillary.

of such non-covalent associations, which arise from relatively weak bonds (hydrogen bonds, etc.), can vary widely, and are reflected by the dissociation constants (K_D) for the complexes formed.³⁰ Several important interactions have been studied using electrospray mass spectrometry, including interactions involving multimeric proteins, receptor-ligand, enzyme-substrate, antibody-antigen, protein-protein, and protein-cofactor complexes.^{25,31-}

40

Of considerable interest to the pharmaceutical industry are the non-covalent binding of antibiotics to their target ligands, usually cell-wall substructures and other biological macromolecules. The vancomycin antibiotics, including vancomycin and ristocetin, bind to the D-alanyl-D-alanine terminus of stem pentapeptides present in bacterial peptidoglycans.⁴¹ This binding inhibits cell-wall biosynthesis, and eventually leads to lysis of the Gram-positive bacterium. Several electrospray/mass spectrometry experiments examining the vancomycin-peptide complex have been conducted, leading to a better understanding of the antibiotic-ligand interaction.^{22,41}

Among the features necessary for the analysis of non-covalent associations using electrospray are proper solution conditions. Typically, ESI/MS studies are carried out in acidic solutions (or alkaline solutions for negative ions), which generally increase the extent of multiple charging.³⁰ However, for the analysis of non-covalent associations, solution conditions approaching those of physiological interest (near neutral pH) are more favorable for preserving the weak structural associations.³⁰ The use of volatile buffer systems such as ammonium acetate replaces acidic additives for electrospray association studies. Other electrospray source conditions are crucial for optimal detection of associations, including lower heated capillary temperatures, lower skimmer-nozzle

voltages, lower analyzer offset voltages, and other parameters which help create gentle conditions and which vary depending on the mass spectrometer used.

A primary consideration in the study of non-covalent complexes is establishing that the species being detected are derived from those in solution.³⁰ One technique for determining the composition of associations is tandem mass spectrometry. MS/MS experiments are very useful for determining whether observed complexes are non-covalent based on their ease of dissociation, and can provide additional information on their composition based on the subunit fragmentations at higher collision energies.³⁰ However, in the cases where CID is not applicable (RF levels during CID with the ion trap would not trap low m/z fragments necessary to determine adduct composition), especially for the larger, singly charged associations analyzed, alternative ion activation techniques are necessary. Infrared multiphoton dissociation is one alternative method useful for the confirmation of the individual components of non-covalent associations, especially when coupled to trapping mass spectrometers like the quadrupole ion trap. Chapter 2 of this thesis will examine the IRMPD capability of the ion trap for the analysis of the vancomycin-peptide complex.

The Quadrupole Ion Trap Mass Spectrometer

History

The quadrupole ion trap has its beginnings with the original patent filed by Paul and Steinwedel in 1956.⁴² Although the two physicists first described the ion trap three years prior, the patent described the three-electrode geometry still used today, and also disclosed the quadrupole mass filter (the 2-D analog of the ion trap). Their fundamental research on ion traps led to the Nobel prize in physics being awarded in 1989 to Paul, for

work on RF ion traps, and Dehmelt, for work on atomic precision spectroscopy. The increased commercial success of the ion trap is primarily attributed to the development of the ion trap as a scanning mass spectrometer, due to the mass selective instability scan developed by Stafford et al. at Finningan Corp.,^{43,44} and the commercial introduction of the first ion trap mass spectrometer in 1983.

The earliest research with the RF ion trap utilized its capability to store ions for long periods of time. Paul, in 1958, was the first person to use a resonance detection method to detect stored ions by applying a small RF voltage between the endcaps and measuring the power absorption of the stored ions.⁴⁵ A similar experiment was conducted by Fisher et al. in 1959. Fisher first showed the mass-selective detection of ions by a power measuring circuit, which achieved unit mass resolution of a series of krypton isotopes.⁴⁶ Also instrumental in the development of mass-selective detection, Rettinghaus et al. acquired the first mass spectrum of residual gas components at a 1 Da/s scan speed by ramping the RF voltage.⁴⁷ In 1968, Dawson and Whetton demonstrated the ability of the ion trap to discriminate between masses using an external detector (electron multiplier).^{48,49} Particularly interesting in these studies was the ability to eject ions from the ion trap to an external detector, which eliminated many of the adverse effects seen with other ion detection methods. These early experiments led Dawson and Whetten in 1971 to develop conditions within the ion trap that were favorable for the storage of ions of only one mass-to-charge ratio (m/z).⁵⁰ This mass-selective storage ability was obtained through a combination of RF and DC voltages applied to the ring electrode. Although these first experiments were primitive by today's standard, they led to further research in the use of the ion trap as a scanning mass spectrometer.

During the 1970's, the quadrupole ion trap was used as a source for quadrupole,⁵⁰⁻⁵³ and sector mass spectrometers.⁵⁴ These experiments led to advances in the understanding of ion/molecule reactions and charge exchange reactions. In 1979, March et al. developed mass-selective isolation and resonant excitation.^{55,56} By applying a supplemental AC voltage (now called a tickle voltage) on each endcap corresponding to a frequency of ion motion, the ion would gain kinetic energy. This led to either ion ejection or collision-induced dissociation (CID). These experiments led to the development of multiple stages of mass spectrometry (MS^n) using the ion trap.

Several developments in the 1980's led to the first commercial quadrupole ion trap instruments. The first was the development of the mass-selective instability scan by Stafford et al. in 1983.^{43,44} By ramping the RF amplitude applied to the ring electrode linearly with time, ions of increasingly higher m/z are sequentially ejected from the ion trap. Stafford et al. improved ion trap performance by introducing a low molecular weight buffer gas (hydrogen or helium).⁵¹ By introducing a light buffer gas at a pressure of 1 mtorr, ion motion is damped through repeated collisions with the neutral gas atoms, eventually focusing the ion cloud to the center of the trap.

The first commercially available quadrupole ion trap was the Finnigan MAT ITD 700 introduced in 1984. This instrument was designed as a low-cost benchtop GC/MS detector. The first fully functional research grade instrument was introduced in 1985. The Finnigan ion trap mass spectrometer (ITMS) offered a number of options not available on earlier instruments, including user-defined scan functions, MS^n capability, EI/CI, and mass isolation. The development of axial modulation in 1988 by Weber-Grabau et al. led to further improvements in mass resolution, and led to the

implementation of mass range extension.⁵⁸ Besides the many improvements made to ion trap performance, the popularity of the ion trap has increased due to the ability to couple to it many external ion sources, including fast-atom bombardment (FAB),⁵⁹ thermospray,⁶⁰ glow discharge,⁶¹ particle beam,⁶² matrix-assisted laser desorption (MALDI),⁶³ and electrospray (ESI).⁶⁴ Several reviews are available outlining these recent advances and presenting a more detailed history of the quadrupole ion trap.⁶⁵⁻⁶⁹

Principles and Theory of Operation

The quadrupole ion trap is a three-electrode device, as shown in figure 2-2, consisting of two endcap electrodes and a ring electrode. To create the quadrupolar electric field, a radio frequency (RF) voltage, V , and a direct current (DC) potential, U , (if any) are applied only to the ring electrode.⁶⁶ Ions within the electric field will possess either stable trajectories and remain trapped, or unstable trajectories and be lost to collisions with electrodes. In addition to the RF and DC voltages applied to the ring electrode, an auxiliary frequency can be applied to the endcap electrodes for resonant excitation and axial modulation during the mass-selective instability scan.

The Mathieu equation is a second-order differential equation that describes the motion of an ion in a quadrupolar field.⁶⁹ The general form of the equation is:

$$\frac{d^2u}{d\xi^2} + (a_u - 2q_u \cos 2\xi)u = 0 \quad (1-1)$$

where u represents r (radial) or z (axial), and $\xi = \Omega t/2$ (Ω is the angular frequency of the RF trapping field and t is the time variable). By performing a series of operations and

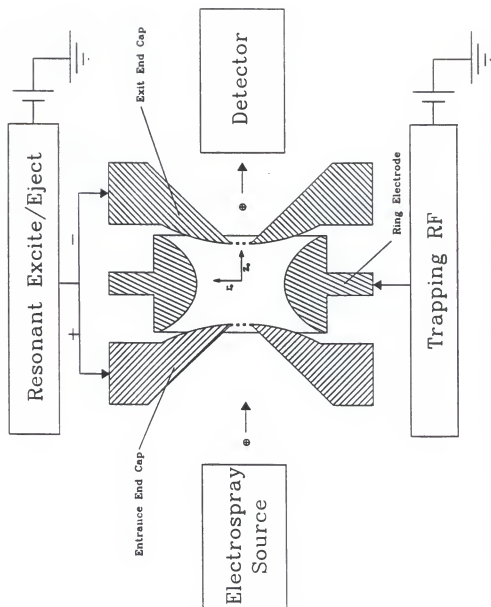


Figure 1-2. Schematic view of the Finnigan MAT ion trap mass spectrometer.

substitutions, the parameters for ion motion in the r and z directions:

$$a_z = -2a_r = \frac{-8eU}{mr_0^2\Omega^2} \quad (1-2)$$

$$q_z = -2q_r = \frac{-4eV}{mr_0^2\Omega^2} \quad (1-3)$$

where m is the ion mass. The Mathieu stability parameters a_z and q_z are directly proportional to the applied DC and RF voltages applied to the ring electrode, respectively, and determine whether ion motion is stable or unstable. A stability diagram specifies the areas within which the axial (z) and radial (r) components of ion motion, respectively, are stable.

Collision-Induced Dissociation

There are two stages of mass spectrometry in MS/MS analyses. The first stage of MS involves the isolation of the precursor ion. Several techniques have been used to perform ion isolation with the quadrupole ion trap. The methods include apex,⁷⁰⁻⁷² two-step,^{73,74} SWIFT (stored waveform inverse Fourier transform),^{75,76} random noise,⁷⁷ forward-reverse scans,^{78,79} and FNF (filtered noise field)⁸⁰ techniques. The apex and two-step isolation techniques are limited due to the high DC voltage required to isolate ions above m/z 600. The SWIFT technique has been shown to be effective for mass isolation above m/z 600, and has also been used in conjunction with IRMPD experiments.⁷ Both the two-step and the forward-reverse scan isolation techniques, first used by Kaiser et al.,⁷⁸ are used for ion isolation in all the IRMPD and CID experiments reported here. The forward-reverse scan method begins with a forward scan (increasing RF) to remove unwanted low mass ions. By ramping the RF voltage, along with a fixed resonant

ejection frequency applied to the endcaps, low mass ions are effectively ejected from the ion trap. For the reverse scan to remove unwanted high mass ions, the RF level is first ramped to a high level. Next, the RF level is ramped in the reverse or downward direction along with a resonant ejection frequency to resonantly eject ions of higher m/z . One unfortunate limitation of this technique is the possibility of unwanted collisional activation of the precursor ion during ion isolation, usually creating more low mass fragment ions. To overcome this problem, the reverse scan is performed before the forward scan; more than one forward or reverse table can also be inserted into the scan function to insure adequate ion isolation.

The most commonly used ion activation method currently used with the quadrupole ion trap is resonant excitation collision-induced dissociation (CID). By applying an AC “tickle” voltage to the endcap electrodes corresponding to an axial frequency component of ion motion, the precursor ion gains kinetic energy, increasing the amplitude of its axial trajectory. This “tickle” voltage can be applied 180° out of phase to the endcaps, producing a dipolar field (utilized on most commercial instruments), or in phase, producing a quadrupolar field. During the resonant excitation step, the precursor ion either is ejected from the trap, or undergoes repeated collisions with the helium buffer gas, converting kinetic energy into internal energy. If enough internal energy is deposited, the precursor ion fragments. A resonance excitation signal is typically 5-30 ms in duration; the amplitude varies from 50-500 mV.

The q_z value during the CID process establishes the RF trapping field during resonant excitation, and therefore determines which ions are effectively trapped. If the q_z

value is not chosen carefully, the precursor ion will be only weakly trapped during resonant excitation and may be ejected from the trap. Typically, a q_z value between 0.2 and 0.3 is chosen for resonant excitation. This value of the RF trapping field allows the precursor ion to gain enough internal energy to fragment, but remain trapped during the resonant excitation period. One adverse effect of choosing a higher q_z value is a higher low mass cutoff. For example, the resonant excitation of the $(M+H)^+$ ion of erythromycin at m/z 734 uses a q_z of 0.2. However, at this q_z , any daughter ion below m/z 162 will not be trapped. Several reports have been published describing the CID process in detail.^{73,74}

Infrared Multiphoton Dissociation

Infrared multiphoton dissociation (IRMPD) is an attractive alternative ion activation technique to CID when performing MS/MS in the quadrupole ion trap. Infrared radiation absorbed by precursor ions trapped within the quadrupole ion trap cause energetic ions to fragment, producing structurally informative daughter ions. The important aspects of infrared absorption are illustrated in figure 1-3. In the figure, M^+ represents an ionic species in its ground state, and $M(E_i)^+$ represents an ion in what is termed the vibrational quasi-continuum. Ions in this region are characterized by the ability to undergo the resonant absorption for a given laser frequency.⁸² Polyatomic ions can be excited to the quasicontinuum by a variety of mechanisms, including thermal excitation, collisional or particle excitation, exothermic chemical reactions, or electronic excitation.⁷⁸ For sufficiently large ions at room temperature, no excitation methods are required since the ions already exist in the quasicontinuum as a result of internal thermal energy content.⁸²

Once in the quasicontinuum, infrared multiphoton excitation leads to either modified bimolecular reaction rates, or unimolecular dissociation. When an excited ion collides with a neutral gas molecule, deactivation processes typically predominate. However, in a few cases, collisions may serve to repopulate the specific rotational levels which are depopulated by laser irradiation, then making absorption of subsequent photons more efficient.⁸² Increases in IRMPD fragmentation efficiencies have even been observed at higher buffer gas pressures.⁸² Unimolecular dissociation is the more common dissociation mechanism with IRMPD if enough photons with energy $h\nu$ are absorbed, and collisional deactivation is minimized.

The IRMPD technique was first used by physicists in the 1960s for spectroscopy experiments on small molecules and ions (MW below 50 Da), but the development of trapping mass spectrometers led to the IRMPD analyses of larger ionic species. The first successful IRMPD experiments were conducted using an ion cyclotron resonance mass spectrometer. In 1979, Beauchamp et al. utilized the IRMPD process for kinetic and reactivity experiments.^{82,83} The first report of a pulsed CO₂ laser for IRMPD studies on an ICR instrument was by Brauman and coworkers, studying the vibrational relaxation of gas-phase ion.^{84,85} Several other researchers were instrumental in the development of photodissociation combined with the ICR, including Richardson et al.,⁸⁶ Eyer,⁸⁷ and Watson.⁸⁸ More recently, electrospray ionization followed by IRMPD in a FTICR instrument has been employed for the complete characterization of large multiply charged proteins and nucleotides.⁸⁹ The same features that make the ICR instrument amenable to IRMPD experiments also apply to quadrupole ion traps. Of particular importance to

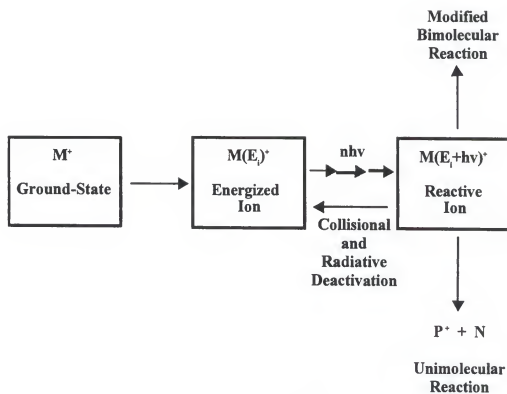


Figure 1-2. Schematic representation of the IRMPD process. Adapted from reference 82.

photodissociation studies is the ability to store ions for long periods of time. This long storage ability is a prerequisite for efficient ion-photon interactions during the IRMPD process. The first successful use of an ion trap for photodissociation studies was by Hughes et al. in 1980.⁹⁰⁻⁹² These first IRMPD experiments examined the dissociation rate constants and photodissociation cross sections of simple alcohols. Louris et al. developed a fiber-optic interface utilizing a pulsed Nd:YAG laser for photodissociation studies on an ion trap.⁹³ However, the first IRMPD studies of electrosprayed ions on a quadrupole ion trap have only recently been reported. Stephenson et al. have examined four classes of biomolecules, including oligosaccharides, oligonucleotides, peptides, and carbohydrate antibiotics.^{5,6} Colorado et al. have also reported IRMPD results for the carbohydrate antibiotics erythromycin and tylosin.⁷

High Performance Liquid Chromatography/Electrospray Mass Spectrometry

It is beyond the scope of this dissertation to discuss the theory and applications of high performance liquid chromatography (HPLC) in any detail. However, several factors need to be considered when combining HPLC with electrospray ionization/mass spectrometry for the analysis of a wide range of biomolecules, including the analysis of antibiotics in this dissertation. While online LC/MS can be relatively straightforward, the solution chemistry necessary to obtain both optimal LC separation and optimal MS performance is not trivial. Typical LC mobile phase additives (sulfates, phosphates, and borates) often added to improve separation characteristics are not suitable for ESI/MS. To obtain optimal sensitivity with LC/ESI/MS analyses, conditions such as solvent choice, pH, and flow rates are all important.

Several suitable additives are useful for pH adjustments for LC/ESI/MS studies. The most common additives⁹⁴ are acetic acid, formic acid, and trifluoroacetic acid (TFA) for positive ion detection, and ammonium hydroxide for negative ion detection (in the 0.1 to 1% range). Several buffers are also useful at a concentration between 10 and 100 μM ,⁹⁴ including ammonium acetate, ammonium formate, triethylamine heptafluorobutyric acid (HFBA), and tetraethyl hydroxide (TEAH). Solvents suitable to LC/ESI/MS applications⁹⁴ include methanol, ethanol, propanol, isopropanol, butanol, acetonitrile, water, acetic acid, formic acid, acetone, dimethylformamide, dimethyl sulfoxide, 2-methoxy ethanol, tetrahydrofuran, and chloroform.

The most common LC separation technique used for the analysis of a wide range of compounds is reverse-phase chromatography. For reverse-phase chromatography to be applicable, the analyte must partition into the non-polar stationary phase. Ions in solution, which are optimal for ESI, lead to poor separation characteristics due to little partitioning into the stationary phase. Several methods have been used to achieve better separation characteristics, including pH adjustments, post-column modifications, and the use of ion-pair chromatography using cationic or anionic additives. Several reviews are available detailing the combination of HPLC with ESI in more detail.⁹⁴⁻¹⁰⁰

CHAPTER 2

IRMPD EXPERIMENTS WITH A CONSTANT PRESSURE OF HELIUM BUFFER GAS USING A PULSED CO₂ LASER

Introduction

Infrared multiphoton photodissociation (IRMPD), or IR photo-induced dissociation (PID), is developing into an important analytical tool for the investigation of gas-phase ion chemistry. When used in conjunction with the quadrupole ion trap, IRMPD has become an effective alternative to collision-induced dissociation (CID) for the activation of ions for MS/MS experiments.³⁻⁷ Electrospray ionization followed by IRMPD allows for the complete characterization of large multiply charged proteins and nucleotides.⁸⁹ For large biomolecules, lower radio frequency trapping voltages (q_z values), applied to the ring electrode of the ion trap during laser excitation, enable the trapping of lower m/z daughter ions. This ability to obtain more detailed structural information is advantageous when compared to resonant excitation CID, where CID efficiency is dependent on the RF trapping voltage applied to the ring electrode.^{2,5,7} This typically requires a higher rf trapping voltage (i.e. higher m/z cut-off value or q_z value) during excitation for efficient fragmentation to occur.^{80,81} Also, kinetic and collisional effects limit the amount of internal energy that can be successfully added to large biomolecules during the CID process. These shortcomings have led researchers to

explore different ion activation techniques for tandem mass analysis, including the combination of photodissociation with ion trapping mass spectrometers.

Several carbohydrate antibiotics have been analyzed with the tandem capabilities of IRMPD combined with the quadrupole ion trap.⁵⁻⁷ Of particular interest to many biochemists is the possibility of selectively activating ions containing glycosidic linkages using IRMPD at the 10.6 μm wavelength where C-O-C bonds absorb. The glycan portions of many proteins and peptides have only recently been discovered as important carriers of information, not useless appendages.^{5,8} However, as more information is obtained on the importance of the glycan moiety, newer analytical techniques are required to successfully identify and sequence glycan units at lower concentrations. A technique that could cleave the intact sugar unit from the core protein without causing fragmentation of the peptide backbone would be invaluable.

Photodissociation combined with the quadrupole ion trap may be one promising analytical technique to obtain sequence and structural information of glycan units. Photodissociation has the potential advantage that energy deposition is well defined with respect to the amount of energy deposited and the internal mode(s) that are initially excited.⁸³ If specific internal modes are excited, i.e. the C-O-C linkage, and fragmentation occurred before the energy redistributed throughout the ion, the potential might exist for targeted bond cleavage in a molecule. In that case, a biochemist using an ion trap could trap the intact glycoprotein, cleave the glycan moiety from the core protein using the appropriate laser energy and wavelength, isolate the intact glycan moiety if it retained a charge, and obtain glycan structure and sequence information from further CID

or PID experiments. Recent work with the selective cleavage of S-S and C-S linkages using different UV wavelengths has suggested the potential for single-photon bond-selective photodissociation from electronic excited states;¹⁰¹ this work has spurred renewed interest in the selective cleavage of other bonds. Unfortunately, the vibrational excitation effected by infrared absorption and the time scale for absorption of multiple infrared photons makes the selective cleavage of glycosidic bonds using the IRMPD technique highly unlikely. Nevertheless, the selective photoabsorption by C-O-C bonds at the 10.6 μm wavelength of the CO_2 laser offers the potential for selective energy deposition into ions containing glycosidic bonds. This selectivity may prove valuable in the analysis of glycoproteins and other carbohydrate biomolecules.

In addition to the possibility of the selective deposition of energy into ions containing glycosidic linkages, the well-defined energy deposition of the photodissociation technique offers the ability to preferentially cleave the weaker non-covalent (versus covalent) bonds of many adducts formed during the gentle electrospray ionization process. Recently, infrared photodissociation has been used as a method to eliminate unwanted non-covalent adducts, particularly sodium and potassium adducts, without the disruption of the covalently bonded core structure.¹⁰² Unwanted adducts from unknown impurities tend to complicate the electrospray spectrum; McLafferty et al. have demonstrated the utility of the IRMPD technique to effectively simplify the ESI mass spectra of large DNA oligomers (50-mer and 100-mer).¹⁰² However, the real potential for IRMPD lies not in the removal of unwanted non-covalent adducts, but in the analysis of receptor-ligand non-covalent complexes that in the past were hard to analyze

directly.³⁰⁻⁴⁰ In the past three years, a wide variety of biologically important non-covalent complexes have been detected using electrospray/mass spectrometry, including enzyme-substrate, duplex DNA, heme-protein, and protein-protein associations.^{25,30-40} Of particular interest to biochemists is the non-covalent binding of antibiotics to their target ligands, typically cell wall substructures and other biological macromolecules. Vancomycin is a complex glycopeptide containing a cross-linked modified heptapeptide backbone with a two-glycan chain. Vancomycin interferes with the biosynthesis of the cell wall of Gram-positive bacteria by forming a strong complex with the peptidoglycan UDP-N-acetylmuramylpentapeptide, specifically the peptide fragment L-lysine-D-alanine-D-alanine.⁴¹

The non-covalent binding of vancomycin with the peptide fragment L-lysine-D-alanine-D-alanine has been examined using electrospray and collision-induced dissociation by Henion et al.²⁵ In this study, collisional activation of the non-covalent adduct served as a confirmatory technique to examine the individual components. However, CID is a non-selective ion activation technique, and often produces unwanted fragmentations.¹⁰³ Furthermore, with trapping instruments the amount of internal energy that can be added to large non-covalent complexes during the CID process is limited. Kinetic and collisional effects limit the amount of internal energy that can be successfully added to large biomolecules, especially when using helium as a buffer gas. A different ion activation technique, specifically IRMPD, would be particularly useful when examining adducts formed with electrospray. Photodissociation can allow control of the energy added to gaseous adduct ions, preferentially cleaving the weaker non-covalent

linkages while leaving the covalent bonds intact. Also, the energy deposited with photon absorption is not a function of the ion mass, in contrast to the CID process in the ion trap.

One other aspect of photodissociation that has not been addressed is the possibility of measuring the binding energies of non-covalent adducts. Several methods have recently been reported for obtaining the relative binding constants of many non-covalent associations with electrospray/mass spectrometry, including the variation of the capillary temperature during the electrospray process.¹⁰⁴ By careful control of the laser energy during the photodissociation process using either a continuous wave or pulsed CO₂ laser, it may be possible to quantify the amount of energy necessary to dissociate these non-covalent adducts.

Several carbohydrate antibiotics have been chosen in this study as probes of the the IRMPD technique using a quadrupole ion trap. The chapter begins with the examination of erythromycin, the first macrolide antibiotic analyzed with the novel electrospray/ion trap instrument built at UF for IRMPD experiments.⁵ Erythromycin consists of a core aglycone ring structure with the glycans desosamine and cladinose covalently attached through glycosidic linkages at C3 and C5. A second macrolide antibiotic examined is tylosin, containing a sixteen-membered aglycone ring with three glycan moieties attached (two in series). The linear carbohydrate neomycin, containing four glycans joined through glycosidic bonds, has also been chosen for IRMPD experiments. A fourth antibiotic, vancomycin, is representative of a class of antibiotics called glycopeptides. Glycopeptide antibiotics consist of a core peptide structure (typically cyclic), with several sugar moieties attached. Lastly, the non-covalent complex

between vancomycin and N,N-diacetyl-D-Lysine-D-Alanine-D-Alanine (Ac_2KAA) will be examined using the gentle ionization technique of electrospray. Once the non-covalent adduct is successfully trapped, the IRMPD technique will be used to confirm the individual components of the adduct.

Experimental

IRMPD Instrument and Procedures

A prototype electrospray quadrupole ion trap mass spectrometer built at the University of Florida was utilized for all IRMPD experiments.^{5,6} The instrument, shown in figure 2-1, consists of a standard Finnigan ITMS ion trap analyzer mounted in a differentially pumped vacuum manifold. A modified Analytica ESI source equipped with a stainless steel heated capillary was coupled to an RF-only octopole ion guide to efficiently transfer electrosprayed ions from the source region to the ion trap analyzer. A tube lens located at the exit orifice of the heated capillary was used as a gate to allow ions through the skimmer and into the octopole region. A 2 cm diameter orifice separates the two manifold regions and allows the exit of the ion guide to be placed directly at the entrance endcap of the ion trap. Although a high pressure (1 mtorr) exists at the octopole entrance, base pressures approaching 9×10^{-7} torr (uncorrected) are obtained in the analyzer region of the manifold (3×10^{-7} when the heated capillary is capped with a septum). The helium inlet system consists of a Granville Philips variable leak valve with 1/8" stainless steel tubing connected to a flange located directly above the ion trap ring electrode (no connecting tube between inlet hole and ion trap). The ion trap contains no quartz spacers (open configuration).

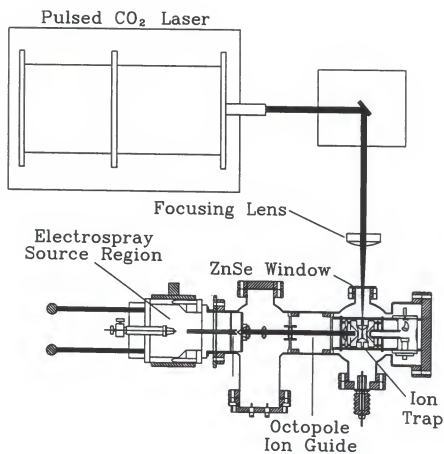


Figure 2-1. Schematic of pulsed CO₂ laser setup for IRMPD combined with an ESI/ion trap instrument.

IRMPD experiments were conducted using a Lumonics Model TE-860-4 pulsed CO₂ laser with a square beam 2.5 cm on a side and a maximum laser energy of 1.0 Joule per pulse. To optimize IRMPD efficiency, the laser beam was focused (lens focal length = 7.5 cm) into the 0.2 cm aperture of the ring electrode. Ion Catcher Software, ICMS, was used to allow software control of the TTL signal for laser firing and acquisition of profile mode mass spectral data.¹⁰⁵

Erythromycin, neomycin, and tylosin were obtained from SIGMA Chemical Co. Vancomycin was obtained from ICN Pharmaceuticals. All antibiotic samples were dissolved in a 1:1 mixture of methanol and water containing 1% acetic acid. Samples were continuously infused using a Harvard Apparatus syringe pump at a flow rate of 3 $\mu\text{L}/\text{min}$ at a concentration of 100 pmol/ μL . The stainless steel electrospray needle used was 100 μm id and held at a potential of +5 kV. The fused silica capillary was 95 μm o.d. and 50 μm i.d. Protonated molecular adducts were obtained for each antibiotic examined.

The timing sequence (scan function) of the ion trap IRMPD experiment using a pulsed CO₂ laser is detailed in figure 2-2. Ion injection occurs at step one in the process. Here, the tube lens gates ions into the octopole region for a predetermined time (typically 50 ms). For ion isolation, both the two-step^{73,74} and the forward-reverse^{78,79} scan methods were used. After isolation, a cool time of 30 ms allows ions to damp to the center of the ion trap before triggering the laser in step four. The laser pulse width is 9 ns with a 50 ms delay between each pulse to allow complete recharging of the laser capacitor. The number of pulses ranged from 5-10 for the efficient IRMPD fragmentation of the

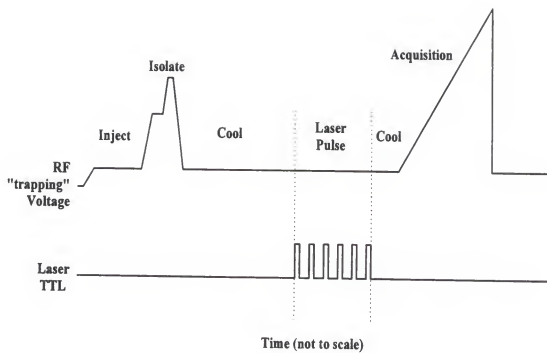


Figure 2-2. Timing diagram for the IRMPD setup using a pulsed CO₂ laser.

protonated antibiotics examined in this study. After laser firing, a second cool time allows ion cooling directly before the detection step, where ions are ejected from the ion trap by ramping the RF voltage applied to the ring electrode (mass-selective instability scan). Mass range extension was utilized for both tylosin and erythromycin using a frequency of 261 kHz.

Electrospray Conditions for the Vancomycin-Ac₂KAA Non-Covalent Complex

N,N-diacetyl-L-Lysine-D-Alanine-D-Alanine (Ac₂KAA) was obtained from SIGMA Chemical Co. Stock solutions of vancomycin and Ac₂KAA were prepared in 10 mM ammonium acetate buffer at a concentration of 3 mg/mL. Solutions of 150 µmol each of antibiotic and ligand were added to polypropylene tubes and diluted to a final volume of 1 mL with 10 mM ammonium acetate. Final solutions were prepared using a 50/50 mixture of 250 µL of the resulting solution and 250 µL acetonitrile. This solution was continuously infused at a flow rate of 3 µL/min.

The first experiments examining the binding of the vancomycin-peptide complex were conducted on a Finnigan MAT LCQ. Subsequent experiments utilizing the IRMPD technique were conducted on a electrospray/ion trap instrument built at the University of Florida. Several parameters of the electrospray source and interface must be optimized to allow the non-covalent complex to remain intact during the electrospray process and transport to the ion trap. These interface conditions are inherently more gentle than normal ESI operating parameters. First, a lower temperature on the heated capillary is necessary for non-covalent associations. For the vancomycin-peptide complex, the capillary was maintained at 150°C. Other variables that create a more gentle transport

condition are the octopole offset and ion trap offset voltages. These two voltages were set to -3V and -5V respectively. By reducing these offset voltages to values lower than normal operating conditions, the kinetic energy of the non-covalent association is reduced when arriving inside the ion trap, reducing the likelihood of CID of these weakly bound complexes.

Results and Discussion

IRMPD of Erythromycin

The large photoabsorption cross section observed for the glycosidic linkage at a wavelength of 10.6 μm makes IRMPD ideal for the structural analysis of carbohydrate antibiotics. The fragmentation nomenclature for carbohydrates as described by Domon and Costello is used throughout.¹⁰⁶ The first antibiotic examined with the prototype electrospray/quadrupole ion trap instrument was erythromycin (MW 733). Erythromycin is a broad-based macrolide antibiotic particularly effective against Gram-positive bacteria.⁸ It acts by inhibiting bacterial protein synthesis by interfering with the 50s ribosome. The structure consists of a core 14-membered aglycone unit with the sugars L-cladinose and D-desosamine attached with glycosidic bonds.

Figure 2-3 displays the IR photodissociation spectra of the protonated adduct of erythromycin at m/z 734 using one, three, and nine laser pulses using a continuous introduction of helium buffer gas. The RF voltage on the ring electrode was set to a q_z value of 0.06 for the parent ion; the overall pressure was maintained at 1×10^{-4} torr (uncorrected) by controlling the helium flow. As shown in figure 2-4, the IRMPD process opens several reaction pathways to produce fragmentation, including dehydration reactions and glycosidic bond cleavages. Using one laser pulse, the daughter ion at m/z

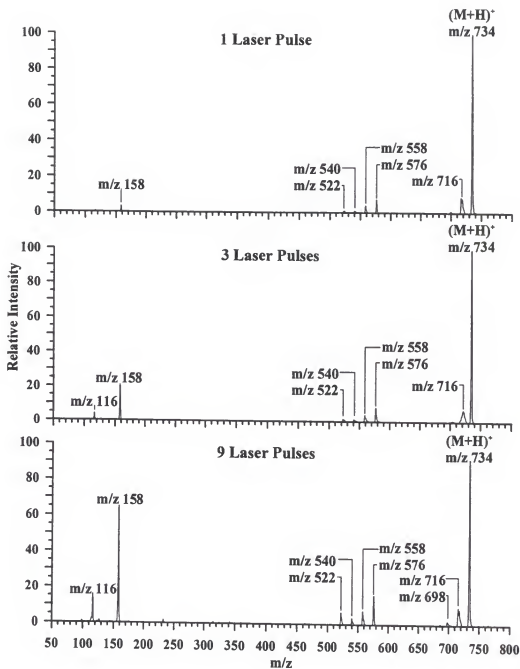


Figure 2-3. IRMPD spectra of the $(M+H)^+$ ion of erythromycin at m/z 734 using one, three, and nine laser pulses.

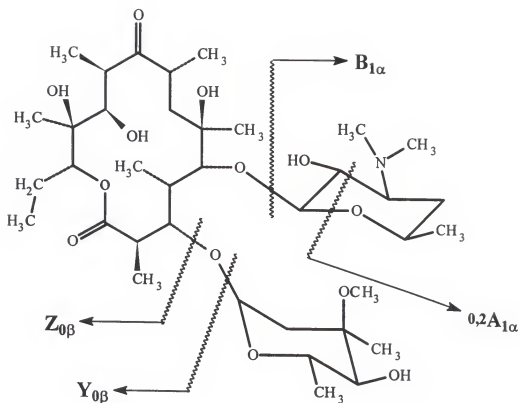


Figure 2-4. The structure of erythromycin showing fragmentation pattern observed with IRMPD.

716 from the loss of one neutral water molecule is produced. Also observed is the ion $(M+H-158)^+$ at m/z 576 ($Y_{0\beta}$) due to the loss of the L-cladinose residue (see structure in Figure 2-4). The ion at m/z 558 could arise from either cleavage of the glycosidic bond on the aglycone side to form the $Z_{0\beta}$ ion, or loss of water from the m/z 576 ion. By selectively ejecting the ion at m/z 576 using a SWIFT waveform, Brodbelt et al. observed the disappearance of the fragment ions at m/z 558 and m/z 540, thereby concluding that the ion at m/z 558 is a dehydration product from the m/z 576 ion.⁷ Barely distinguishable, successive losses of water from the m/z 558 ion yield the ions at m/z 540 and m/z 522. Several low-mass ions are observed due to the low RF storage conditions ($q_z = 0.06$) during the ion activation step; these fragmentations are charge-site driven for the ring opening of the D-desosamine sugar.⁵ Direct cleavage on the right side of the glycosidic bond (with accompanying hydrogen transfers) of the D-desosamine sugar is indicated by the fragment ion at m/z 158 ($B_{1\alpha}$). Increasing the laser irradiance to three laser pulses (3 J energy) increases the overall intensity of the fragment ion population. The ion observed at m/z 116 is the $^{0.2}A_{1\alpha}$ fragment ion due to ring cleavage of the D-desosamine (see structure in figure 2-4). Increasing to nine laser pulses (9 J energy) provides a further increase in photodissociation efficiency of protonated erythromycin: 4% for one pulse versus 20% for nine pulses. The daughter ion observed at m/z 698 is due to the loss of two water molecules from the protonated adduct.

The first IRMPD experiments using the combined electrospray/ion trap system were obtained with a constant background of helium buffer gas. The analyzer manifold pressure was maintained at 1×10^{-4} torr (uncorrected) for optimal operation of the ion trap

analyzer. Unfortunately, the helium introduced inside the ion trap to increase trapping and detection efficiencies can collisionally deactivate photoexcited ions during the IRMPD process. Lowering the helium pressure within the analyzer region increases the extent of photodissociation observed, but signal intensities are drastically reduced and are often undetected at pressures necessary for effective IRMPD (as will be shown in chapter 3, optimal conditions are 2×10^{-6} torr or lower).

IRMPD of Neomycin

Neomycin is an antibiotic complex composed of neomycin A, B, and C. Neamine is the two-sugar component of neomycin A (see structure figure 2-5). The other two-sugar component of neomycin A is called neobiosamine B. Neomycin B and C are stereoisomers containing four sugar moieties arranged in a linear structure. The antibiotic was first obtained from cultures of *Streptomyces fradiae* and subsequently from other species, and is active against both Gram-positive and Gram-negative bacteria, mycobacteria, and actinomycetes.¹⁰⁷ In medicine it has found use in the treatment of bacterial infections of the eye and skin, and also for the sterilization of the intestine.

The IRMPD spectrum of the $(M+H)^+$ ion of neomycin A at m/z 615 obtained using six laser pulses at a pressure of 1×10^{-4} torr (uncorrected) is shown in figure 2-5. The base peak in the spectrum is the $(M+H)^+$ ion at m/z 615. Loss of the terminal saccharide from either end gives the daughter ion at m/z 455. Cleavage here with charge retention on the terminal sugar moiety produces the fragment ion at m/z 163. Cleavage of the centrally located glycosidic linkage with charge retention on the neamine portion produces the ion at m/z 323. Further dissociation of the m/z 323 ion with cleavage of the

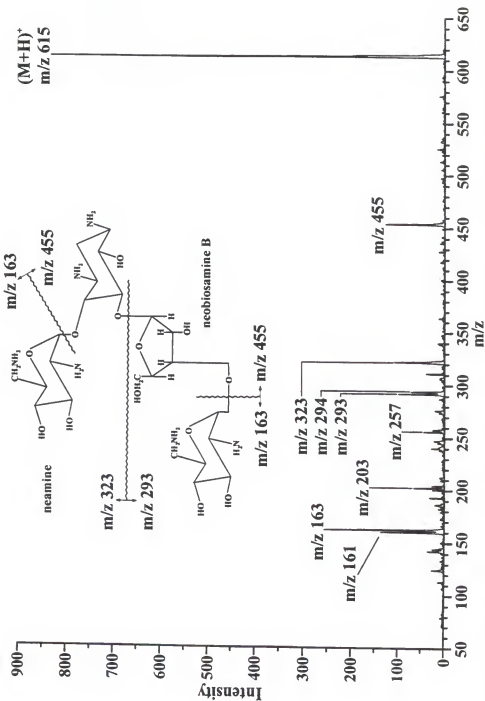


Figure 2-5. IRMPD spectrum of the $(M+H)^+$ ion of neomycin A using nine laser pulses with a constant background pressure of helium buffer gas (1×10^{-4} torr).

glycosidic linkage and charge retention on the second sugar produces the daughter ion at m/z 161. The fragment ion at m/z 293 is due to cleavage at the central glycosidic bond with charge retained by the neobiosamine B portion of neomycin. Cleavage of both terminal sugar moieties leaving the two central glycans intact gives the fragment ion at m/z 295.

IRMPD of Tylosin

Tylosin is a macrolide antibiotic (16-membered ring) containing the three glycans mycarose, desosamine, and mycinose. The IRMPD spectrum of the $(M+H)^+$ ion of tylosin at m/z 916 using fifteen laser pulses is presented in figure 2-6. Again, helium was continuously infused at an uncorrected ion trap pressure of 1×10^{-4} torr. The base peak in the spectrum is the $(M+H)^+$ ion at m/z 916. Loss of the terminal mycarose sugar gives the Y_1 fragment at m/z 772. The additional loss of one water molecule produces the fragment ion at m/z 756. Cleavage of the aglycone-desosamine glycosidic linkage generates the Y_0 fragment ion at m/z 598. If the charge is retained by the amine group of desosamine, the B_2 daughter ion is produced at m/z 319. The B_2 ion can also lose the mycarose sugar generating the abundant daughter ion at m/z 174. The loss of one water molecule from this fragment gives the ion at m/z 156. Due to the low RF voltage used during ion activation ($q_z=0.07$ for m/z 916), other low mass fragment ions are observed below m/z 174. The presence of the single basic site (the tertiary amine on the desosamine glycan) dictates that the most abundant daughter ions (m/z 174, 319, 772, etc) retain this amino glycan and thus the charge. Loss of the desosamine sugar (Z_0) and the mycarose sugar on the other side of the aglycone ring generates the charged macrolide

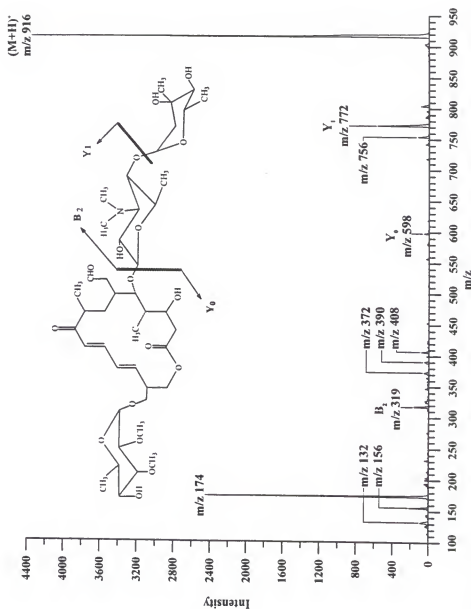


Figure 2-6. IRMPD spectrum of the $(M+H)^+$ ion of tylosin using fifteen laser pulses with a constant background pressure of helium buffer gas (1×10^{-4} torr).

ring at m/z 408. Consecutive losses of water give the daughter ions at m/z 390 and m/z 372. Overall, the IRMPD spectrum of tylosin gives enough structurally significant daughter ions to help elucidate the structure, even though daughter ion signal intensities are low (base peak is the parent ion). Improving the efficiency of the photodissociation process would increase the signal intensities of the fragment ions obtained for tylosin and other macrolide antibiotics.

IRMPD of Vancomycin

Vancomycin is a glycopeptide antibiotic containing a cross-linked heptapeptide backbone extensively modified; it is effective against Gram-positive bacteria.²² Vancomycin binds to a peptidoglycan precursor of the bacterial cell wall, inhibiting cell wall growth. The IRMPD spectrum of the $(M+H)^+$ ion of vancomycin at m/z 1450 is shown in figure 2-7 using ten pulses from the pulsed CO_2 laser and a manifold pressure of 1×10^{-4} torr (uncorrected). The $(M+H)^+$ ion is shown at m/z 1450. Cleavage of the terminal amino sugar gives the daughter ion at m/z 1306 ($Y_{1\beta}$). The loss of both sugar moieties gives the daughter ion at m/z 1144 ($Y_{0\beta}$). A daughter ion of low intensity at m/z 1333 occurs due to the sequential loss of the covalently linked N-methyl-D-leucine residue and ammonia from the protonated vancomycin. Although this IRMPD spectrum of vancomycin includes several structurally significant daughter ions, the photodissociation efficiency is low (8%), as evidenced by the large signal obtained for the parent ion at m/z 1450. The photoexcitation process is being damped by the high pressure of helium necessary for optimal ion trap performance.

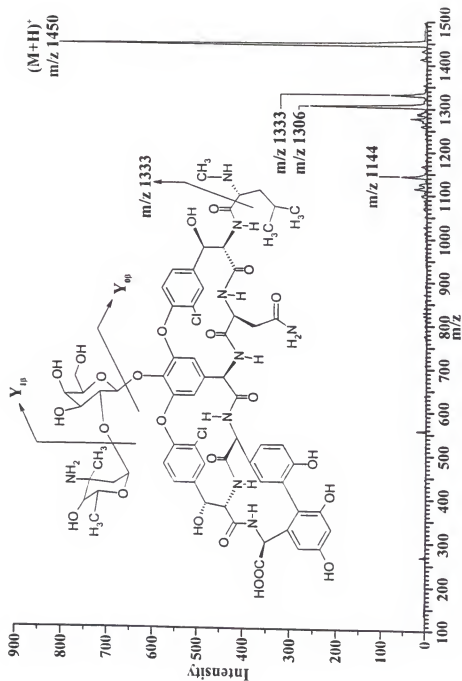


Figure 2-7. IRMPD spectrum of the $(M+H)^+$ ion of vancomycin using ten laser pulses with a constant background pressure of helium buffer gas (1×10^{-4} torr).

Analysis of the Vancomycin-Ac₂KAA Non-Covalent Complex

Figure 2-8 displays the electrospray mass spectrum of the vancomycin-Ac₂KAA complex obtained using the Finnigan LCQ using a 1:1 molar ratio of antibiotic:ligand. Gentle electrospray source and interface conditions were maintained for optimal signal intensity for the singly charged (Vancomycin+Ac₂KAA+H)⁺ complex ion at m/z 1822. The (Vancomycin+Ac₂KAA+Na)⁺ ion is seen at m/z 1844. The doubly charged complex, (Vancomycin+Ac₂KAA+2H)²⁺, can also be seen at m/z 912. The individual components of the vancomycin-peptide complex can also be observed. The (Ac₂KAA+H)⁺ ion can be seen at m/z 373, and the (Ac₂KAA+Na)⁺ ion is the base peak at m/z 395. The sodium adduct of the peptide dimer, (Ac₂KAA+Ac₂KAA+Na)⁺, can be observed at m/z 767, and the ion at m/z 783 is the potassium adduct of the peptide dimer, (Ac₂KAA+Ac₂KAA+K)⁺. If the ion source conditions were not "gentle", the signal intensity for the complex ion at m/z 1822 and m/z 912 were low. One interesting feature of the vancomycin-peptide complex is the observation of the singly charged ion at m/z 1822. Henion et al. only observed the doubly charged complex ion at m/z 912.²⁵ This could possibly be attributed to the tuning parameters used on the triple quadrupole system.

IRMPD of the Vancomycin-Ac₂KAA Non-Covalent Complex

The structural confirmation of the vancomycin-peptide complex can be obtained utilizing the photodissociation capabilities of the electrospray/ion trap instrument built at UF. The electrospray mass spectrum of the vancomycin-peptide complex was similar to that obtained using the Finnigan LCQ. IRMPD should allow the analyst better control of

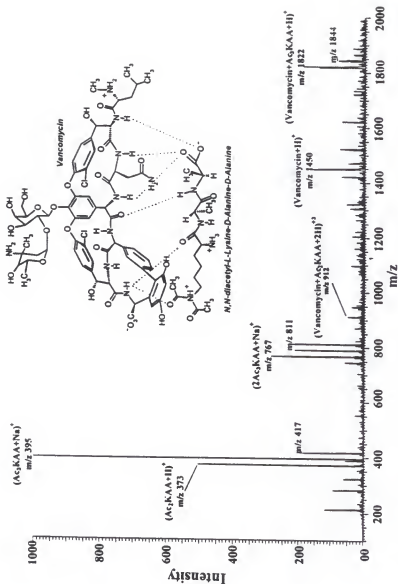


Figure 2-8. Electrospray mass spectrum of the vancomycin-Ac₂KAA non-covalent complex at m/z 1822.

the energy added to the complex, i.e. the number of laser pulses, to allow the preferential cleavage of the weaker non-covalent linkages (hydrogen bonds), while leaving the covalent bonds of the receptor-ligand complex intact. Using thirteen pulses of the CO₂ laser, the IRMPD spectrum of the vancomycin-Ac₂KAA complex is shown in figure 2-9. The singly protonated complex is first isolated at m/z 1822 (see figure 2-9 A). The IRMPD spectrum (figure 2-10 B) shows primarily the protonated vancomycin at m/z 1450. Only a low intensity vancomycin fragment ion (m/z 1306) can be observed; the vancomycin fragment ion at m/z 1144 is not observed. The singly protonated Ac₂KAA peptide (m/z 373) was not observed in this experiment (ion trap was scanned low enough, but m/z 373 signal not observed), presumably due to charge retention by vancomycin during the IRMPD experiment. This experiment demonstrates the capability of the IRMPD process to assist in the analysis of high molecular weight non-covalent associations when using electrospray ionization/quadrupole ion trap mass spectrometry. Current experiments are underway utilizing CID of the vancomycin-peptide complex, and comparisons between the two ion activation techniques for the analysis of non-covalent adducts will prove valuable.

Conclusions

Due to the high photoabsorption cross-section of the C-O-C linkage of the glycosidic bond, IRMPD of several carbohydrate antibiotics using a novel electrospray/ion trap instrument with a pulsed CO₂ laser has been quite successful. The (M+H)⁺ ions of the macrolide antibiotics, erythromycin and tylosin produced several structurally significant daughter ions using 13 to 15 laser pulses. Due to the low RF

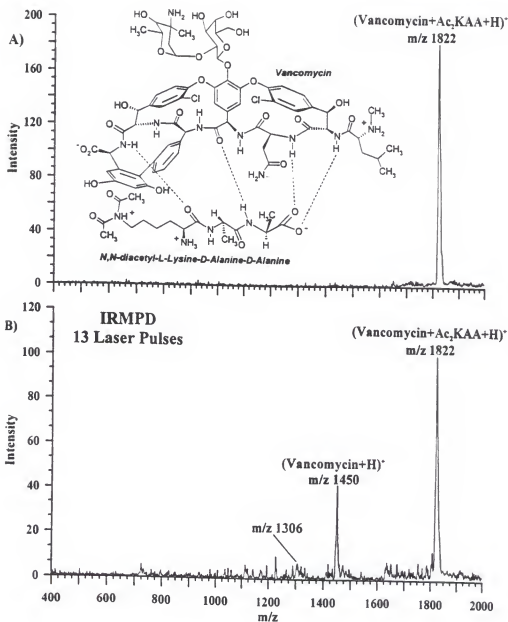


Figure 2-9. (A) Electrospray mass spectrum of the vancomycin-Ac₂KAA non-covalent complex, with isolation of the adduct at m/z 1822. (B) IRMPD of the complex using thirteen laser pulses.

voltages applied to the ring electrode during laser irradiation (low q_z values of 0.06 for the parent ion), many of the low m/z daughter ions were efficiently trapped and detected with the IRMPD technique. In contrast, CID in the ion trap would require that the parent ions be held at higher q_z values to prevent ejection of the parent ion during resonant excitation; the higher q_z values would preclude trapping of lower m/z daughter ions. Similar results were obtained with the antibiotics neomycin and vancomycin.

The IRMPD results using an ion trap for the antibiotics studied were promising, but the overall efficiency of the IRMPD process was low (4% to 20%). In every IRMPD spectrum acquired at a pressure of 1×10^{-4} torr with the pulsed CO_2 laser, the parent ion was more intense than any daughter ions. The cause of the inefficient photodissociation process is the helium buffer gas required for optimal ion trap performance. A constant infusion of helium at an uncorrected pressure of 1×10^{-4} torr collisionally deactivates many of the photoexcited ions during the IRMPD process.

One interesting application of the IRMPD technique is the analysis of non-covalent adducts. The well-defined energy deposition of the photodissociation technique offers the ability to preferentially cleave the weaker non-covalent bonds of many adducts formed during the gentle electrospray process. The CID ion activation technique also is applicable to the analysis of non-covalent adducts, but there is a greater possibility of cleaving covalent bonds within the complex. The application of IR photodissociation for non-covalent adducts was effectively demonstrated using the vancomycin- AC_2KAA non-covalent adduct at m/z 1822.

CHAPTER 3

INVESTIGATIONS OF COLLISIONAL QUENCHING OF PHOTOEXCITED IONS DURING THE IRMPD PROCESS

Introduction

Recently, progress has been made towards increasing the efficiency of the photodissociation process inside the ion trap. With the application of an axial, quadrupolar dc pulse to the endcaps, ions were radially focused and forced to fall within the confines of the laser beam, thereby increasing photodissociation efficiency.¹⁰⁸ Increased IRMPD efficiency has also been achieved in our laboratory by incorporating three spherical concave mirrors into the ring electrode of the ion trap.¹⁰⁹ This modified configuration allowed eight laser passes across the radial plane of the ring electrode producing higher photoabsorption efficiencies. The observed photodissociation efficiency for protonated diglyme was 90% or higher, considerably higher than the single pass optical design. Although these improvements have increased the efficiency of the IRMPD process, collisional damping of photoexcited ions within the ion trap remains an obstacle to the routine use of IRMPD as an analytical tool.¹⁰⁹

For photoinduced fragmentation to occur efficiently, the rate of photon absorption must be faster than the rate of collisional deactivation.⁸² Helium buffer gas introduced into the ion trap to increase trapping efficiency and mass resolution can collisionally deactivate photoexcited ions during the IRMPD process. Lowering the buffer gas

pressure reduces collisional quenching, but significantly reduces overall ion trap performance (sensitivity and resolution).^{57,110} Recently, our group has explored the use of short pulses of helium buffer gas for efficient trapping and cooling of ions.^{109,111} Pulsed valves have been used extensively as analyte gas inlets with internal ionization and in ion molecule reaction experiments.¹¹²⁻¹¹⁵ Pulsed valves have also been used extensively to increase CID efficiencies in the Fourier transform mass spectrometer (FTMS) while maintaining ultra-high-vacuum conditions at other points in the scan function.¹¹⁶ However, the use of pulsed valves as a helium buffer gas inlet is finding more applications.^{109,111} A short pulse of helium buffer gas injected directly before detection damps ions to the center of the ion trap, thereby increasing the transmission of ions through the exit endcap to the detector, thus increasing ion detection efficiency.¹¹¹ With internal ionization, a short pulse of helium before ionization efficiently traps ions once formed while maintaining low pressures during the RF ramp during data acquisition.¹⁰⁹ This same approach can be used with ions injected from external ionization sources such as electrospray and matrix-assisted laser desorption (MALDI). With external ionization sources coupled to ion traps, a short pulse of helium can be employed directly before ions are gated into the trapping region. The pulse of helium effectively traps the injected ions; one group has even reported increases in sensitivity using a pulsed inlet of helium when compared to using a constant pressure of helium buffer gas.¹¹⁷

To eliminate the problem of collisional deactivation during photodissociation in the studies presented here, a scan function is used which introduces a short pulse of helium into the ion trap directly before ion injection. Once ions are efficiently trapped, a

delay time allows most of the helium to be pumped away before laser firing. After a sufficient irradiation period for either the pulsed or cw laser, a second helium pulse is introduced before detection to increase ion detection efficiency. Using a dual pulsed introduction of helium for IR photodissociation studies dramatically reduces collisional quenching during the photoexcitation process and improves photodissociation efficiencies significantly.

This chapter presents the first data obtained with a novel scan function employing two short pulses of helium before ion injection and ion detection to increase photodissociation efficiencies. The first section of this chapter discusses IRMPD efficiency as a function of helium pressure in the analyzer region. This is followed by several ion injection experiments using short pulses of helium before injection and detection to examine the utility of the method. Next, some of the first IRMPD data of antibiotics using a pulsed inlet of helium with a continuous-wave CO₂ laser are presented.

Experimental

A prototype electrospray ion trap mass spectrometer built at the University of Florida was utilized for all IRMPD studies.^{5,6} The instrument consists of a standard Finnigan ITMS ion trap analyzer mounted in a differentially pumped vacuum manifold. A modified Analytica electrospray source equipped with a stainless steel heated capillary was coupled to an RF-only octopole ion guide (12 in. long) to efficiently transfer electrosprayed ions from the source region to the ion trap analyzer. The analyzer region and octopole are shown in figure 3-1.

Erythromycin was obtained from SIGMA Chemical Co., and vancomycin was

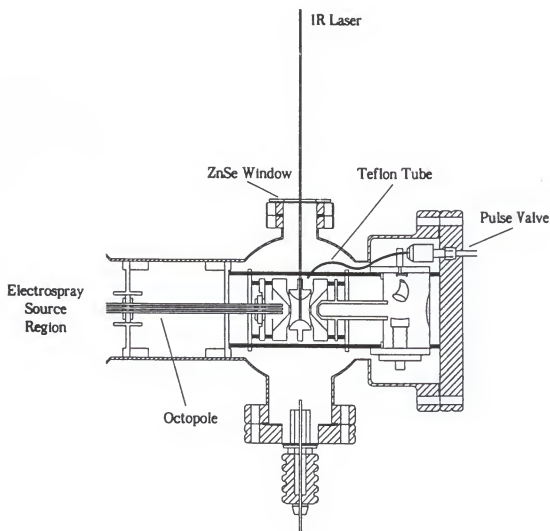


Figure 3-1. Schematic of the ion trap IRMPD setup for the pulsed introduction of helium buffer gas.

obtained from ICN Pharmaceuticals. All antibiotic samples were dissolved in 1:1 methanol:water containing 1% acetic acid. Samples were continuously infused at a rate of 3 $\mu\text{L}/\text{min}$ at a concentration of 100 $\text{pmol}/\mu\text{L}$. IRMPD experiments were conducted using both continuous wave (cw) and pulsed CO_2 lasers operating at a wavelength of 10.6 μm . An Apollo model 570 cw laser with a beam diameter of 1 cm (unfocused) and a maximum power output of 50 W and a Lumonics model TE-860-4 pulsed CO_2 laser with a square beam 2.5 cm on a side and a maximum laser energy of 1.1 Joule per pulse were used. To optimize IRMPD efficiency with the pulsed CO_2 laser, the laser beam was focused (lens focal length = 7.5 cm) onto the 0.2 cm aperture of the ring electrode.

The pulsed valve (General Valve Series 9) shown in figure 3-1 was directly mounted to the back flange behind the multiplier region. The exit orifice diameter of the pulsed valve was 0.006 in. A 1/8 in o.d. Teflon tube attached to the tip of the pulsed valve delivered helium buffer gas directly outside the ring electrode (with ring spacers removed). Ion catcher software, ICMS, developed at UF was used to allow software control of the TTL signal and acquisition of profile mode spectra.¹⁰⁵ A pulsed valve controller (built at the University of Florida) accepted the external TTL signal generated by the ITMS electronics and triggered the pulsed valve. A second TTL signal triggered laser firing. All manifold pressures were measured by a Bayard-Alpert ionization gauge mounted on the vacuum chamber directly below the ion trap, and are uncorrected. The mass range of the ITMS was extended by lowering the axial modulation frequency during data acquisition to 161 kHz and 111 kHz respectively. The axial modulation voltage was maintained at 4700 mV.

Results and Discussion

IRMPD Efficiency as a Function of Pressure

Figure 3-2 shows the effect of helium buffer gas on the photodissociation efficiency of protonated erythromycin at m/z 734 using a cw CO_2 laser. For this experiment helium was introduced into the analyzer region with a variable leak valve to yield a constant pressure during the entire scan function. The photodissociation efficiency (P_D) for a given reaction is defined as the fraction of the parent ion population photodissociated over a given exposure time (t) for a specified laser irradiance:

$$P_D = 1 - (I / I_0) \quad (3-1)$$

where I is the signal intensity of the dissociating ion (m/z 734 for erythromycin) measured at the end of the exposure period (100 ms), and I_0 is the signal intensity after the same time period without irradiation. Measurements for I and I_0 were an average of 5 consecutive analytical scans (laser off followed by laser on) with corresponding error bars defined as standard deviation of the mean. As expected, at higher overall pressures the photodissociation efficiency is drastically reduced. At a pressure of 1.5×10^{-5} torr and below, the photodissociation efficiency for erythromycin is at least 96%. Increasing the pressure slightly in the analyzer region to 5.5×10^{-5} torr drastically reduces the photodissociation efficiency to approximately 5%. A steep transition occurs over the pressure range of $1.5\text{--}3.0 \times 10^{-5}$ torr. The plot indicates that effective IRMPD for protonated erythromycin using a cw laser occurs at a pressure at or below 1.5×10^{-5} torr.

Unfortunately, normal operation of the quadrupole ion trap requires a constant background pressure of helium buffer gas (typically 10^{-3} torr, which corresponds to

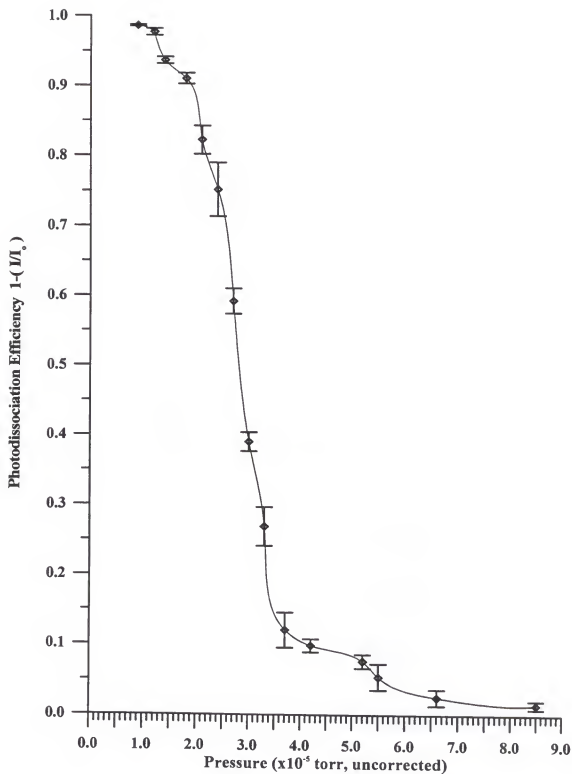


Figure 3-2. IR photodissociation efficiency for the (M+H)⁺ ion of erythromycin at *m/z* 734 as a function of helium buffer gas pressure in the ion trap region using an irradiation time of 100 ms (energy = 1.8 J).

approximately 10^{-4} torr uncorrected). The efficient trapping of ions injected from an electrospray source into the ion trap requires a helium buffer gas to damp ion motion (kinetic energy) for improved trapping efficiency. Also, the efficient ejection and detection of ions to achieve acceptable sensitivity and resolution dictates the use of a buffer gas. Obviously, for IRMPD studies with a quadrupole ion trap, a technique is needed to reduce the overall pressure inside the analyzer region during the laser irradiation step, while maintaining adequate buffer gas pressure for efficient ion injection and detection.

Ion Injection Using Short Pulses of Helium

One method to overcome the effect of collisional quenching of photoexcited ions at higher pressures is to reduce the pressure during photoexcitation by a pulsed introduction of helium buffer gas at certain points during the scan function. A short pulse of helium directly before ion injection effectively damps ion motion, allowing the efficient trapping of injected ions. A second pulse of helium directly before ion ejection and detection allows the efficient detection of trapped ions. Before resuming IRMPD studies, several experiments were conducted without laser irradiation to evaluate the performance characteristics of an ion trap scan function incorporating pulsed introduction of helium. Figure 3-3 compares the ion trap performance for the $(M+H)^+$ ions of caffeine and the peptide MRFA (Met-Arg-Phe-Ala) using (A) constant background of helium buffer gas (1×10^{-4} torr, uncorrected) and (B and C) pulsed introduction of helium. Figure 3-3 B shows that a 30 ms pulse of helium directly before ion injection (gating) gives two times more signal for caffeine at m/z 195 with poor mass resolution; however, the ion

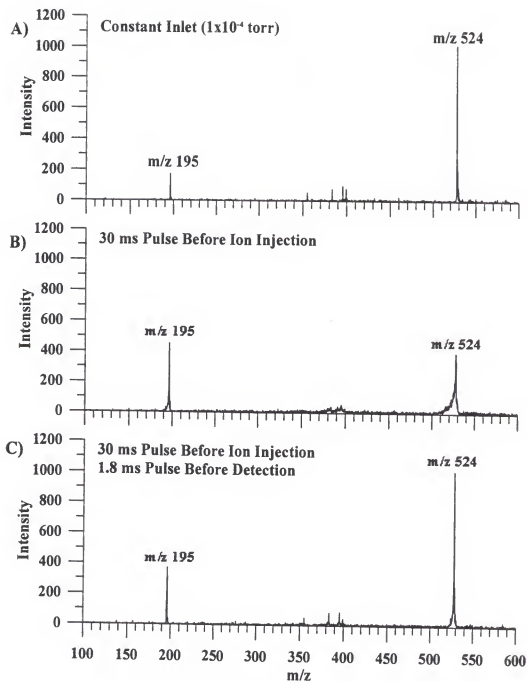


Figure 3-3. A comparison of the different helium inlet methods for ion injection of the $(M+H)^+$ ion of caffeine at m/z 195 and the peptide MRFA at m/z 524.

signal for the singly charged protonated adduct of MRFA at m/z 524 is poorly resolved and weak (400 counts compared with 1100 counts with constant helium). By adding a second pulse of helium directly before ion detection, the ion trap sensitivity and resolution are improved over the single pulse method. Figure 3-3 C shows the signals for caffeine and MRFA using both a 30 ms pulse before ion injection and a 1.8 ms pulse before ion detection. For the $(M+H)^+$ ion of MRFA at m/z 524, the signal intensity is comparable with that obtained with a constant background pressure of helium. For caffeine, the protonated adduct signal at m/z 195 has increased two-fold.

It should be pointed out, however, that the mass resolution obtained with the dual pulsed inlet of helium is reduced when compared with that obtained with a constant inlet of helium. This may result from either a buffer gas pressure that is too high or too low, or from inadequate time for the ions to be cooled to the center of the trap. Decreasing the pulse width on the second pulse led to improved resolution, but decreased sensitivity. Several experiments were conducted in an attempt to improve the mass resolution by placing a cool period between the pulse of helium and the scan acquisition step. Results were inconclusive; more experiments are needed to characterize the effects on the resolving power of the experimental parameters of the pulsed helium inlet technique.

For the IRMPD analysis of biomolecules ionized by electrospray, including the antibiotics used in this study, the ions of interest are typically above m/z 500. Several ions were examined with the pulsed inlet of helium to see if larger ions were efficiently trapped and detected. Figure 3-4 presents the signal intensity for the $(M+H)^+$ ion of erythromycin at m/z 734 using a pulsed inlet of helium. The lower trace was obtained by

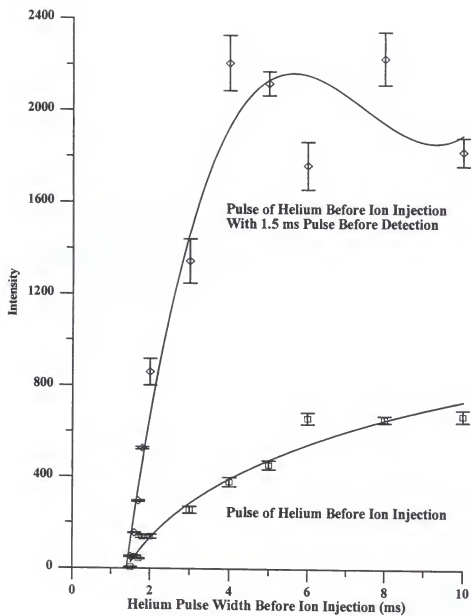


Figure 3-4. Erythromycin signal intensity for the $(M+H)^+$ ion at m/z 734 as a function of helium buffer gas pulse width before ion injection.

varying the width of a single helium pulse directly before ion injection. At pulse widths below 1.2 ms, the mechanical response of the pulsed valve actuator was too slow to allow the valve to open. For the $(M+H)^+$ ion of erythromycin, the signal intensity appears to level off past a 6 ms pulse of helium. This intensity was comparable with that obtained with a constant pressure of helium of 1×10^{-4} torr (uncorrected). The addition of a second pulse of helium before detection, along with the first pulse before ion injection, enhanced the ion detection efficiency significantly. The upper trace in figure 3-4 was obtained using a 1.5 ms pulse of helium before detection, while varying the helium pulse before ion injection. Here, the signal intensity peaks around a 4 ms pulse width before ion injection. The addition of the second 1.5 ms pulse of helium significantly improves sensitivity at m/z 734 over the use of just one pulse of helium before ion injection, as evidenced by the four-fold increase in ion signal intensity over the one-pulse method.

For studies of electrosprayed ions above m/z 1000, the polymer ultramark 1621 was used. This polymer mixture gives a series of singly charged ions separated by 100 Da between m/z 900 and m/z 2000. For this reason ultramark is commonly used as a calibration compound for electrospray. The effect of a pulsed introduction of helium on the intensities of the peptide MRFA and ultramark ions is displayed in figure 3-5. The top spectrum shows the results obtained with a constant inlet of helium at a pressure of 1×10^{-4} torr (uncorrected). Figure 3-5 B shows the signal intensity obtained with an 8 ms pulse of helium only before ion injection. Here, both the signal intensity and mass resolution are poor. By adding a 1.8 ms pulse of helium before ion ejection and detection along with the 8 ms pulse before ion injection, acceptable ion trap performance is

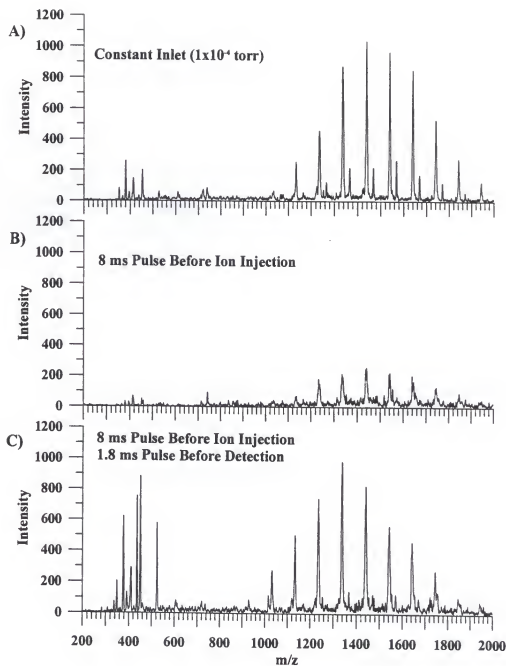


Figure 3-5. A comparison of the different helium inlet methods for the peptide MRFA and the polymer ultramark 1621.

obtained. When compared with the results using a constant inlet of helium, the sensitivity obtained is equivalent, but the mass resolving power is reduced. The series of ions below m/z 600 are from the $(M+H)^+$ ion of the peptide MRFA. To obtain the high m/z ions of ultramark, the tube lens (gate lens) located at the exit orifice of the heated capillary was increased to its maximum value of +129 V. However, raising this gating potential creates harsh electrospray source conditions, and collision-induced dissociation (CID) occurs. Several fragments from MRFA are observed, including the c_3 ion at m/z 452, the b_3 ion at m/z 438, the x_3 ion at m/z 421, the a_3 ion at m/z 410, the y_3 ion at m/z 393, and the z_3 ion at m/z 378. One interesting feature observed in figure 3-5 C is that the signal intensities of these low mass fragment ions have increased two-fold when compared with those obtained with a constant inlet of helium. The dual pulsed helium inlet technique appears to favor low m/z ions.

Scan Function Using a Pulsed-Inlet of Helium for IRMPD Studies

After examining the performance characteristics of the pulsed helium inlet technique, an IRMPD scan function was developed incorporating a pulsed inlet of helium producing 10x lower overall pressures (1×10^{-5} torr) within the analyzer region during laser firing. The timing sequence of the IRMPD experiment is shown in figure 3-6 for a continuous wave CO₂ laser. A variable-length pulse of helium (typically 6 to 10 ms) placed directly before the ion injection event effectively traps injected ions. Experiments placing a delay time between the helium pulse and ion injection indicated no delay was necessary for optimal performance. After isolation of the parent ion via either two-step or forward-reverse scans, a 30 ms delay time was included to allow for removal of any

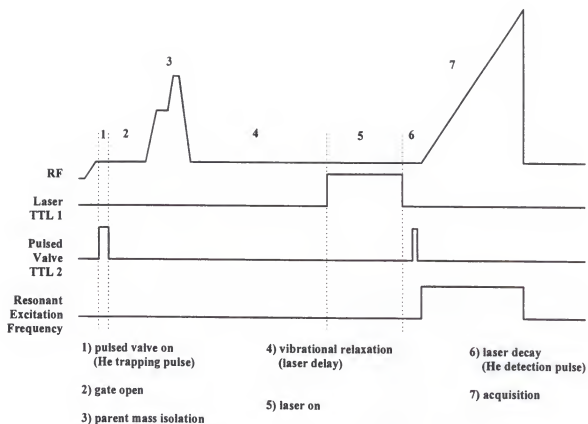


Figure 3-6. IRMPD timing diagram incorporating a dual pulsed introduction of helium buffer gas.

excess internal energy by radiational and/or collisional cooling. The parent ion was then irradiated with the cw CO₂ laser with laser pulse length being sample dependent (50 ms to 500 ms). After a sufficient irradiation period, a 1.5 ms helium detection pulse was used to improve detection efficiency prior to the mass-selective instability scan over the range of m/z 50 to 800 for erythromycin and m/z 100 to 1500 for vancomycin.

IRMPD of Erythromycin Using a Pulsed-Inlet of Helium

The IRMPD spectrum of the $(M+H)^+$ ion of erythromycin using a dual pulsed inlet of helium with an irradiation time of 100 ms (energy = 1.8 J) is shown in figure 3-7. By reducing the pressure during the IRMPD experiment, the photodissociation efficiency (sum of daughter ion signals / parent ion signal) has increased above 90% when compared to 5-15% with the pulsed CO₂ laser experiment at high pressure (figure 2-3). A small peak is observed for the remaining $(M+H)^+$ parent ion at m/z 734. Successive losses of water give the ions at m/z 716 and m/z 698. Cleavage of the cladinose sugar gives a low intensity peak at m/z 576 ($Y_{0\beta}$). Successive dehydration reactions of this daughter ion gives the ion at m/z 558, m/z 540, and m/z 522. Several ions are observed in the range m/z 300 to m/z 500. The ions at m/z 482 and m/z 464 correspond to successive losses of water from the daughter ion at m/z 500. A definitive identification of these ions has not been made to date, although the possibility of a ring-opening reaction involving the aglycone ring is being examined. The IRMPD experiment appears to be opening reaction pathways that are not typically observed with either low-energy or high-energy CID. The daughter ion at m/z 158 is the $B_{1\alpha}$ ion from cleavage of the desosamine glycan. A low intensity daughter ion at m/z 116 is observed for the $^{0,2}A_{1\alpha}$ ion. Overall,

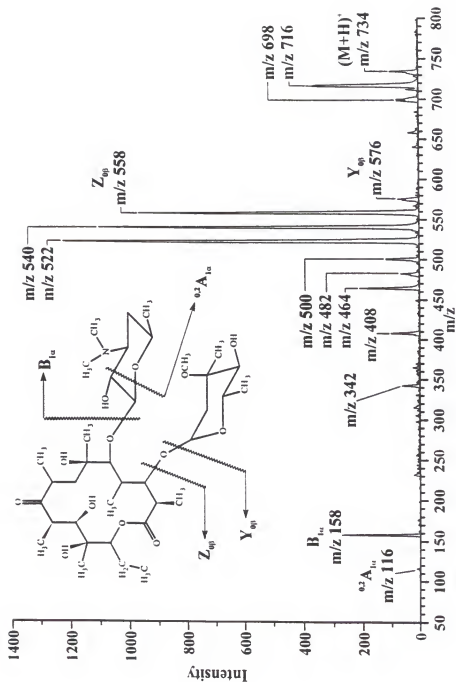


Figure 3-7. IRMPD spectrum of the $(M+H)^+$ ion of erythromycin at m/z 734 using a dual pulsed introduction of helium with an irradiation time of 100 ms (energy=1.8J).

the pulsed helium inlet scan function significantly increases the photodissociation efficiency for the protonated adduct of erythromycin.

IRMPD of Vancomycin Using a Pulsed-Inlet of Helium

Figure 3-8 displays the IRMPD spectrum of the $(M+H)^+$ ion of vancomycin with the dual pulsed helium introduction method and a 450 ms irradiation time (energy = 8.1 J). The parent ion in the spectrum is the singly charged protonated adduct at m/z 1450. Cleavage of the terminal glycan gives the $Y_{1\beta}$ daughter ion at m/z 1306. Cleavage of both glycans gives the $Y_{0\beta}$ daughter ion at m/z 1144. A daughter ion of low intensity at m/z

1333 occurs due to the sequential loss of the covalently linked N-methyl-D-leucine residue and ammonia from the protonated vancomycin. The IRMPD results with a dual pulsed inlet of helium with the cw laser were comparable with the results obtained with the pulsed laser at higher pressures. Several experiments are currently being conducted to identify conditions optimal for obtaining low mass daughter ions (below m/z 700) from the singly and doubly protonated adducts of vancomycin.

Conclusions

• The efficient trapping and detection of injected ions with a quadrupole ion trap requires the use of a buffer gas of helium to damp ion motion. However, this relatively high pressure of helium buffer gas can collisionally quench photoexcited ions during the IRMPD process. For effective IRMPD experiments using the ion trap, a technique must be developed to lower the overall pressure in the analyzer region during laser irradiation. In the past the only alternative was to lower the helium pressure to levels that are optimal

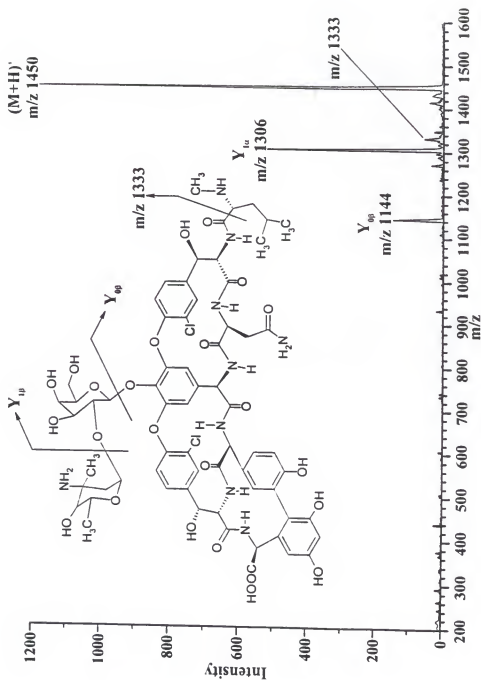


Figure 3-8. IRMPD spectrum of the $(M+H)^+$ ion of vancomycin at m/z 1450 using a dual pulsed introduction of helium with an irradiation time of 450 ms (energy=8.1 J).

for photodissociation, but ineffective for ion trapping and detection. One technique to improve IR photodissociation efficiencies in the ion trap, demonstrated here, incorporates a pulsed introduction of helium into the analyzer region before and after laser firing. Several experiments with protonated caffeine, the peptide MRFA, and ultramark 1621 indicate a dual pulsed introduction of helium gives comparable sensitivity when compared to a constant inlet of helium, although with decreased mass resolution. By pulsing helium into the ion trap at specific times in the scan function, before ion injection and before ion detection, enough time is allowed for most of the helium to be pumped away before the photoactivation step of the IRMPD experiment. This pulsed introduction of helium produces increased photodissociation efficiencies (above 90%).

CHAPTER 4

IRMPD EXPERIMENTS WITH A PULSED INTRODUCTION OF HELIUM BUFFER GAS USING A PULSED CO₂ LASER

Introduction

Recently, research has led to a better understanding of the way antibiotics inhibit bacterial cell growth. Antibiotics have three mechanisms of action: inhibition of protein synthesis, inhibition of cell wall construction, and inhibition of DNA replication.¹⁰⁷ However, microbes have developed defenses for each mechanism by producing proteins that destroy, block, decoy, or remove the offending antibiotic.¹ Researchers are countering this threat of antibacterial resistance with an arsenal of new agents, including the modification of older, proven antibiotics. For example, biochemists have replaced the attachment site of the cladinose sugar of erythromycin with a keto group to obtain a more potent antibiotic with little bacterial resistance.¹ However, modifying the structure of older antibiotics even slightly requires a sensitive and accurate analytical technique to ensure that the modification was successful. One technique that has allowed the structural elucidation of many labile, high molecular weight antibiotics is electrospray ionization combined with tandem mass spectrometry.^{25,31-40} The triple quadrupole tandem mass spectrometer has long been the workhorse of the pharmaceutical industry, but the recent development of the quadrupole ion trap has spurred interest in the ion trap's ability to perform tandem mass analyses at a lower cost.

With a quadrupole ion trap, resonant excitation collision-induced dissociation (CID) is the method of choice for ion activation during MS/MS experiments. During resonance excitation, trapped ions undergo energetic collisions with helium buffer gas molecules.² The isolated parent ion fragments if enough internal energy is deposited by the multiple-collision process. A prerequisite for efficient CID, however, is that the RF trapping field during the resonant excitation process must be chosen between a q_z value of 0.2 and 0.3 for optimal results.⁸⁰ This value of the RF field allows the parent ion to gain enough internal energy to fragment yet remain trapped during the CID process. However, using a q_z value of 0.2 to 0.3 creates unfavorable trapping conditions for many of the low mass daughter ions created. For example, using a q_z value of 0.2 during the CID of the $(M+H)^+$ ion of tylosin at m/z 916, ions below m/z 200 have unstable trajectories and are not detected, and valuable structural information is lost. Also, CID efficiencies decrease when examining large molecular weight biomolecules. Kinetic and collisional effects limit the amount of internal energy that can be successfully added to large biomolecules, especially when using helium buffer gas.^{61,114} One solution to this limitation is to use heavier buffer gases such as argon or xenon during resonant excitation; limitations to this method have been reported, however, including discharges in the ion trap.¹¹⁴

To aid the bioanalytical mass spectrometrists in drug discovery, new approaches to the structural elucidation of antibiotics using the quadrupole ion trap are attractive. Several alternative ion activation techniques have been explored, including electron beam excitation^{118,119} and surface-induced dissociation.^{120,121} However, for the activation of

large biomolecules, photodissociation, specifically infrared multiphoton dissociation (IRMPD), when used in conjunction with trapping mass spectrometers is developing into an important analytical tool for structural determination.^{5-7,89} One drawback of the IRMPD technique when combined with the quadrupole ion trap is collisional quenching of photoexcited ions by the helium buffer gas used to improve ion trapping and detection efficiencies. Modifications are required to the current ion trap operating conditions to increase the IRMPD fragmentation efficiencies for the analysis of many large biomolecules, including carbohydrate antibiotics.

This chapter will highlight recent improvements to the IRMPD ion activation technique when combined with the quadrupole ion trap. As shown in Chapter 3, to reduce the helium pressure in the analyzer region during laser irradiation, a unique scan function incorporating a dual pulsed inlet of helium was developed for IRMPD studies. The first data collected using a pulsed CO₂ laser will be presented. Next, a comparison will be made with data collected at higher analyzer pressures using a continuous introduction of helium buffer gas. Finally, a comparison between the IRMPD of several antibiotics will be made with the conventional ion activation technique of collision-induced dissociation.

Experimental

The instrumental configuration using a prototype electrospray (ESI) / quadrupole ion trap mass spectrometer is shown in figure 3-1. A General Valve Series 9 pulse valve was used throughout the studies shown here. IRMPD experiments were conducted using a Lumonics Model TE-860-4 pulsed CO₂ laser with a square beam diameter of 2.5 cm

and a maximum laser energy of 1.0 Joule per pulse. To optimize IRMPD efficiency, the laser beam was focused (lens focal length = 7.5 cm) into the 0.2 cm aperture of the ring electrode. Ion Catcher Software, ICMS, was used to allow software control of the TTL signal for laser firing and the pulse valve.¹⁰⁵

Erythromycin, neomycin, and tylosin were obtained from SIGMA Chemical Co. Vancomycin was obtained from ICN Pharmaceuticals. All antibiotic samples were dissolved in a 1:1 mixture of methanol and water containing 1% acetic acid. Samples were continuously infused at a flow rate of 3 $\mu\text{L}/\text{min}$ at a concentration of 100 $\text{pmol}/\mu\text{L}$. Protonated molecular adducts were obtained for each antibiotic examined.

The timing sequence (scan function) of the ion trap IRMPD experiment using a two-pulsed introduction of helium with a pulsed CO_2 laser is detailed in figure 4-1. Ion injection occurs at step one in the process. Here, the tube lens gates ions into the octopole region for a predetermined time. For ion isolation, both the two-step and the forward-reverse scan methods were used. After isolation, a short cool time of 30 ms allows ions to dampen to the center of the ion trap before triggering laser firing in step four. The laser pulse width is 9 ns with a 50 ms delay between each pulse to allow complete recharging of the laser capacitor. The number of pulses ranged from 5-10 for the efficient IRMPD fragmentation of the antibiotics examined in this study (lower number required at lower pressures). After laser firing, a second cool time allows ion cooling directly before the detection step where ions exit the ion trap by ramping the RF voltage applied to the ring electrode (mass-selective instability scan).

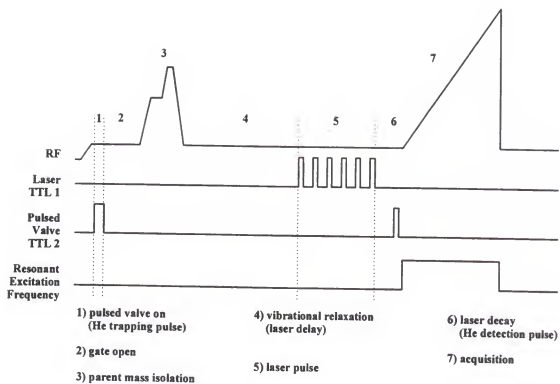


Figure 4-1. IRMPD timing diagram using a dual pulsed inlet of helium with a pulsed CO_2 laser.

Results and Discussion

IRMPD of Erythromycin Using a Pulsed-Inlet of Helium

The first carbohydrate antibiotic examined using the dual pulsed inlet of helium buffer gas with a pulsed CO₂ laser was erythromycin. Figure 4-2 displays the IRMPD spectrum of the (M+H)⁺ ion of erythromycin at m/z 734 obtained using 6 laser pulses (energy = 6 J) at a reduced pressure of 1x10⁻⁵ torr during laser firing. Easily noticable in the IRMPD spectrum are the intense daughter ion signals relative to the precursor ion at m/z 734. Again, the ions at m/z 716 and m/z 698 are successive losses of water from the protonated adduct. A signal of low intensity is obtained for the Y_{0β} ion at m/z 576. However, the signal intensity for the daughter ion at m/z 558 from the dehydration reaction of the Y_{0β} ion is very intense. The daughter ions at m/z 540 and m/z 522 are successive losses of water from the m/z 558 ion. A series of ions first observed during IRMPD at the reduced ion trap pressures with the continuous-wave laser are also detected with 6 pulses of the pulsed CO₂ laser. The series consists of daughter ions at m/z 232, m/z 342, m/z 408, m/z 464, m/z 482, and m/z 500. The daughter ion at m/z 500 loses successive water molecules to produce the ions at m/z 482 and m/z 464. These ions are apparently the product of a ring-opening reaction of the 14-membered lactone ring (at the ether linkage). A tentative structural assignment has been made for the daughter ion at m/z 232, which appears to be a product of the lactone ring opening. Two aglycone ring cleavages at C4 and C6 with charge retention on the desosamine sugar produces an ion at m/z 232. A more definitive structural identification of these ions has yet to be made, but the possibility of gaining structural information from antibiotics not normally obtained

with low-energy and high-energy CID has attracted the attention of many researchers in the pharmaceutical industry. Of considerable importance to the structural elucidation of erythromycin are several low-mass daughter ions. Glycosidic bond cleavage at the D-desosamine sugar produces an intense signal at m/z 158 for the B_{1a} ion. Cleavage of the same glycosidic linkage (with accompanying hydrogen transfer) of D-desosamine to the left of the O atom is indicated by the C_{1a} daughter ion at m/z 174. Also observed are two charge-site fragmentations for the ring opening of the D-desosamine sugar producing the ions at m/z 100 ($^{2,5}A_{1a}$) and m/z 116 ($^{0,2}A_{1a}$). Overall, the reduced ion trap pressures obtained with a pulsed introduction of helium buffer gas during laser firing and ion activation have improved the IRMPD fragmentation efficiency considerably.

The improvement in IRMPD efficiency obtained with a dual pulsed inlet of helium using a quadrupole ion trap can clearly be seen in figure 4-3. Here, an IRMPD comparison for the $(M+H)^+$ ion of erythromycin at m/z 734 is made between a constant inlet of helium and a pulsed inlet of helium using 9 laser pulses (energy = 9 J). Figure 4-3 A displays the IRMPD results at a constant pressure of 1×10^{-4} torr (uncorrected); photodissociation efficiency was 20%. The molecular ion in the mass spectrum is the protonated precursor ion at m/z 734. Daughter ions obtained are of low signal intensity and no ions are observed in the range of m/z 200 to m/z 500. However, with a dual pulsed introduction of helium buffer gas, the IRMPD spectrum of the $(M+H)^+$ ion of erythromycin obtained at a reduced overall pressure (1×10^{-5} torr during laser firing) is significantly improved; photodissociation efficiency was above 90%. Of particular note

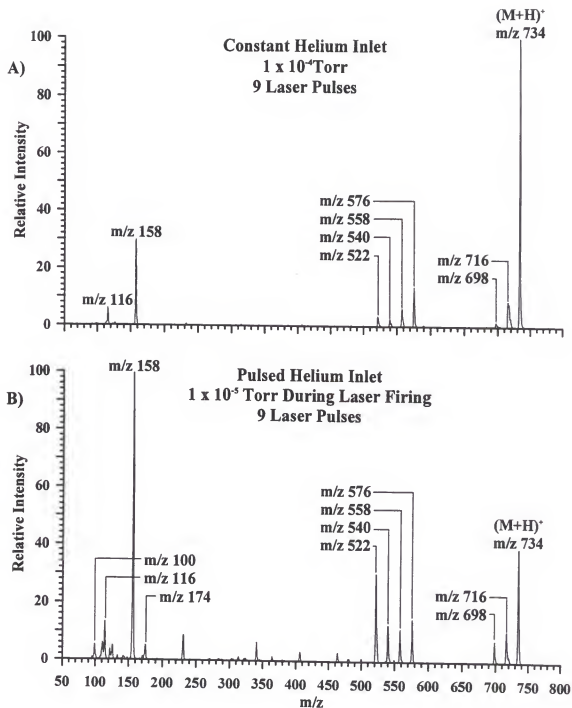


Figure 4-3. Comparison of the IRMPD spectra of the $(M+H)^+$ ion of erythromycin at m/z 734 using two different helium inlets.

is the intense signal intensity obtained for the $B_{1\alpha}$ ion at m/z 158, and the daughter ions obtained at m/z 100 ($^{2,5}A_{1\alpha}$) and m/z 174 ($C_{1\alpha}$), which are not observed in the IRMPD spectrum obtained at higher pressures.

Collision-Induced Dissociation Versus IRMPD of Erythromycin

For the MS/MS process involving collision-induced dissociation, fragmentation is dependent on the trapping conditions established by the RF voltage applied to the ring electrode during ion activation. The kinetic energy of the parent ion of interest increases with the absorption of power from a supplementary resonant excitation (kick) AC voltage. Ion dissociation, ejection, and/or ion-molecule reactions can occur during the entire process, which collectively limit CID efficiencies obtainable with the quadrupole ion trap. Using IR photodissociation for MS/MS analyses, however, eliminates the competition between resonance ejection and fragmentation of the parent ion. Here, fragmentation is dependent on the absorption cross section of the ion, which is dependent primarily on the presence of individual functional groups which absorb at the laser wavelength. At the wavelength of 10.6 μm , the IR absorbing functional groups are the C-O-C linkage of glycosidic bonds, the amide bond of peptides and proteins, and the phosphodiester bond of oligonucleotides and DNA/RNA oligomers.^{5,6,89}

Figure 4-4 displays a comparison of the CID and IRMPD spectra for the $(M+H)^+$ ion of erythromycin at m/z 734. Using a q_z of 0.15 (lower than the normal q_z value of 0.2 to aid in trapping low m/z daughter ions; LMCO = 122), the CID spectrum of the $(M+H)^+$ ion of erythromycin is shown in figure 4-4 A. The CID spectrum is highlighted by several high mass daughter ions at m/z 716, m/z 698, m/z 576, m/z 558, m/z 540, and m/z

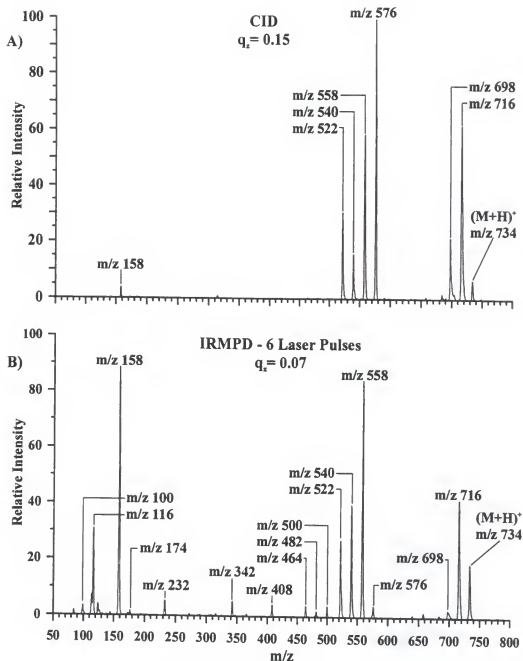


Figure 4-4. Comparison between CID and IRMPD for the $(M+H)^+$ ion of erythromycin at m/z 734. (A) CID spectrum acquired at a pressure of 1×10^{-4} torr, $q_z = 0.15$, frequency = 44.2 kHz, and amplitude = 3V. (B) IRMPD spectrum with dual pulsed inlet of helium at a $q_z = 0.07$.

522. The only low-mass daughter ion observed is the $B_{1\alpha}$ ion at m/z 158. Several experiments were conducted at even lower RF trapping voltages (q_z values between 0.1 and 0.15) to improve the trapping of low mass daughter ions during the CID process, but proved ineffective due to the inability to hold the precursor ion at m/z 734 within the trap during resonant excitation. The IRMPD spectrum obtained with a pulsed inlet of helium with 6 laser pulses is shown in figure 4-4 B (same spectrum is presented in figure 4-2). The same high m/z daughter ions are observed in the IRMPD spectrum. However, due to the lower RF trapping voltages which can be applied to the ring electrode ($q_z = 0.07$), more structurally significant low mass daughter ions are obtained. Of particular note is the intense signal intensity obtained for the $B_{1\alpha}$ ion at m/z 158, and the daughter ions obtained at m/z 100 ($^{2,5}A_{1\alpha}$) and m/z 174 ($C_{1\alpha}$), which are not observed in the CID spectrum. Also, the series of daughter ions between m/z 200 and m/z 500 are not observed in the CID spectrum (nor have they been observed with high-energy CID on sector instruments).¹²²

IRMPD of Neomycin Using a Pulsed-Inlet of Helium

The IRMPD spectrum of the $(M+H)^+$ ion of neomycin at m/z 615 obtained using a dual pulsed inlet of helium with 6 laser pulses is shown in figure 4-5. The structure for neomycin showing several fragmentation pathways observed in IRMPD (and CID) is displayed in figure 4-6. The base peak in the spectrum is the parent ion at m/z 615. Loss of the terminal saccharide from either end gives the daughter ion at m/z 455. Successive losses of water gives the ions at m/z 437, 419, and 401. Cleavage at the same point with charge retention on the terminal sugar moiety produces the fragment ion at m/z 161.

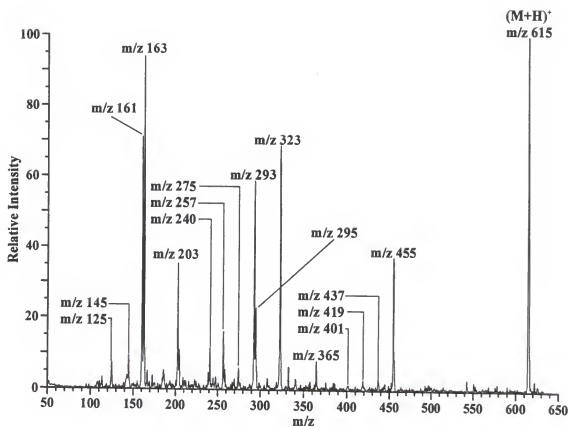


Figure 4-5. IRMPD spectrum of the $(M+H)^+$ ion of neomycin using a dual pulsed inlet of helium and 6 laser pulses at a q_z of 0.07.

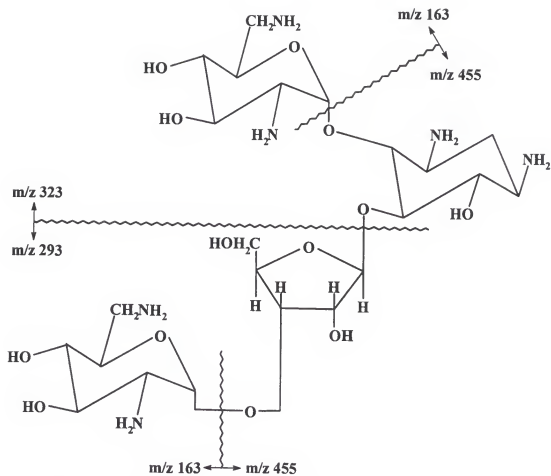


Figure 4-6. The structure of neomycin showing fragmentations patterns.

Cleavage of the centrally located glycosidic linkage with charge retention on the neamine portion produces the ion at m/z 323. Further dissociation of the m/z 323 ion, with cleavage of the glycosidic linkage and charge retention on the second sugar produces an intense daughter ion signal at m/z 163. Dehydration of the ion at m/z 163 produces the daughter ion at m/z 145; dehydration of the ion at m/z 161 produces the ion at m/z 143. The daughter ion at m/z 143 undergoes further dehydration to produce the ion at m/z 125. The fragment ion at m/z 293 is due to cleavage at the central glycosidic bond with charge retained by the neobiosamine B portion of neomycin. Successive losses of water give the daughter ions at m/z 275, m/z 257, and m/z 240. Cleavage of both terminal sugar moieties leaving the two central glycans intact gives the fragment ion at m/z 295. Overall, the IRMPD fragmentation efficiency for the $(M+H)^+$ ion of neomycin has been increased using a dual pulsed inlet of helium. At the reduced pressures obtained (1×10^{-5} torr), dehydration reactions predominate the IRMPD spectrum. Due to the non-selectivity of the IRMPD process, many of these daughter ions formed from dehydration reactions are secondary daughter ions formed from primary daughter ions which undergo further ion activation during laser irradiation.

A comparison of the IRMPD results using different helium inlets for the $(M+H)^+$ ion of neomycin at m/z 615 is presented in figure 4-7. Figure 4-7 A displays the IRMPD spectrum using 6 laser pulses with a continuous introduction of helium buffer gas (1×10^{-4} torr, uncorrected). Very little fragmentation is obtained at these normal ion trap operating pressures; photodissociation efficiency was approximately 15%. However, incorporating a dual pulsed introduction of helium buffer gas into the ion trap scan

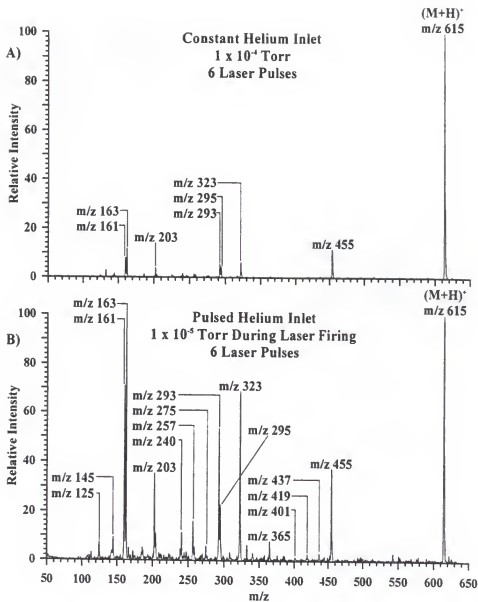


Figure 4-7. Comparison of the IRMPD spectra of the $(M+H)^+$ ion of neomycin at m/z 615 using two different helium inlets.

function produces an ion trap pressure of 1×10^{-5} torr (uncorrected) during laser irradiation. The IR photodissociation efficiency is significantly enhanced (above 90%). Of particular note are the increased signal intensities for several of the daughter ions, including the ions at m/z 161, 163, 203, 293, 295, 323, and 455. Several daughter ions which were not observed at higher pressures during the IRMPD experiment are observed at the lower pressures with a pulsed inlet of helium, including the ions at m/z 125, 145, 240, 257, 275, 365, 401, 419, and 437.

Collision-Induced Dissociation Versus IRMPD of Neomycin

Figure 4-8 displays a comparison of the CID and IRMPD spectra for the $(M+H)^+$ ion of neomycin at m/z 615 (same spectrum presented in figures 4-5 and 4-7 B). The MS/MS spectrum using collision-induced dissociation at a q_z of 0.15 (LMCO = 103) is shown in figure 4-8 A. The base peak for the CID spectrum is the daughter ion at m/z 455. Several daughter ions that have been identified with IRMPD are also present in the CID spectrum, including the ions at m/z 161, 163, 203, 257, 293, 295, 323, 437, and 455. When comparing the CID spectrum with that obtained from IRMPD at low pressures, more useful fragmentation information is obtained using photodissociation using a pulsed inlet of helium with 6 laser pulses ($q_z = 0.07$). Of particular note are several of the daughter ions from dehydration reactions that are more prominent in the IRMPD spectrum. The daughter ion signals for the ions at m/z 275, 257, and 240, formed through successive water losses of the ion at m/z 293, are very intense in the IRMPD spectrum (the ions at m/z 275 and 240 are not observed in the CID spectrum). Also, the daughter ions at m/z 419, 401, 143, 145, and 125 are not observed in the CID spectrum, but are

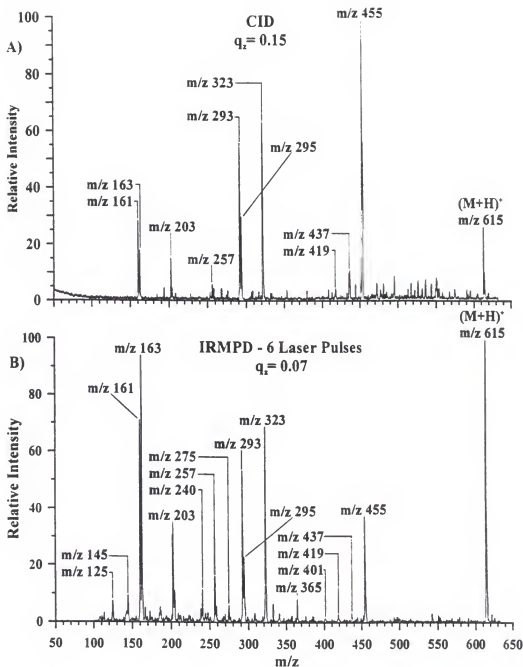


Figure 4-8. Comparison between CID and IRMPD of the $(M+H)^+$ ion of neomycin at m/z 615. (A) CID spectrum acquired at a pressure of 1×10^{-4} torr, $q_z = 0.15$, frequency = 71 kHz, and amplitude = 2.2 V. (B) IRMPD spectrum acquired using a dual pulsed inlet of helium at a $q_z = 0.07$.

prominent in the IRMPD spectrum.

IRMPD of Tylosin Using a Pulsed-Inlet of Helium

The IRMPD spectrum of the $(M+H)^+$ ion of tylosin at m/z 916 using a pulsed inlet of helium with 10 laser pulses (energy = 10 J) is presented in figure 4-9. The overall analyzer manifold pressure during laser irradiation was 1×10^{-5} torr (uncorrected). The base peak in the spectrum is the parent ion at m/z 916. Loss of the terminal mycarose sugar gives the Y_1 fragment at m/z 772. Cleavage at the other side of the glycosidic bond gives the fragment ion at m/z 756. Cleavage of the aglycone-desosamine glycosidic linkage generates the Y_0 fragment ion at m/z 598. If the charge is retained by the amine group of desosamine, the B_2 daughter ion is produced at m/z 319. The B_2 ion can also lose the mycarose sugar generating the intense daughter ion at m/z 174. The loss of water from this fragment gives the ion at m/z 156. Due to the low RF voltage used during ion activation ($q_z=0.07$), other low mass fragment ions are observed below m/z 174. Loss of the desosamine sugar (Z_0) and the mycarose sugar on the other side of the aglycone ring generates the charged macrolide ring at m/z 408. Consecutive losses of neutral water give the ions at m/z 390 and m/z 372. Overall, the IRMPD spectrum of tylosin using a pulsed inlet of helium increases the photodissociation efficiency to above 90%.

This improvement in IRMPD fragmentation efficiency can be clearly seen in figure 4-10. Here, a comparison between the IRMPD spectra of the $(M+H)^+$ ion of tylosin using two different helium inlets is made. Figure 4-10 A presents the IRMPD results using a constant introduction of helium at an uncorrected pressure of 1×10^{-4} torr with 10 laser pulses. The base peak in the spectrum is the parent ion at m/z 916; the most intense

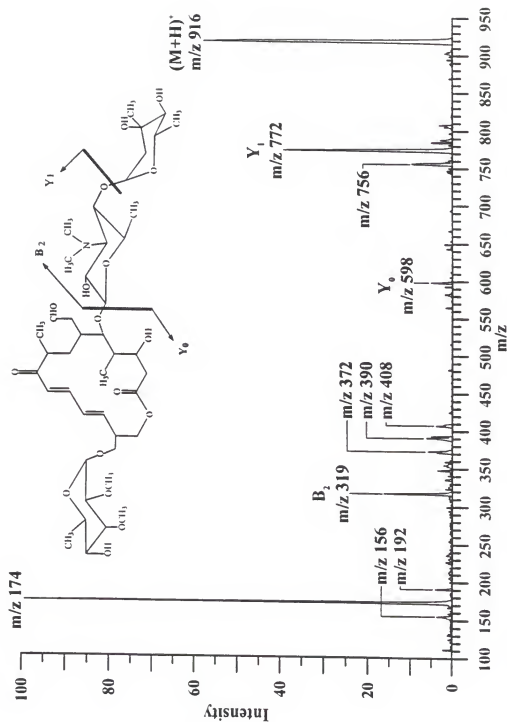


Figure 4-9. IRMPD spectrum of the $(M+H)^+$ ion of tylosin at m/z 916 using a dual pulsed introduction of helium with 10 laser pulses.

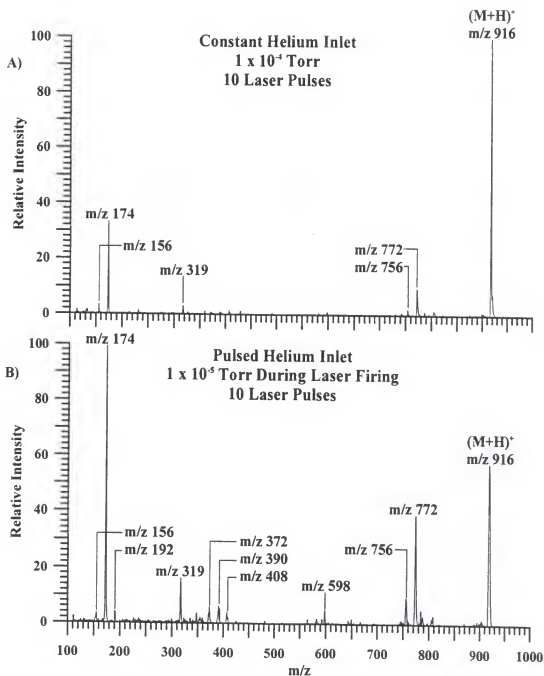


Figure 4-10. Comparison of the IRMPD spectra of the (M+H)⁺ ion of tylosin at m/z 916 using two different helium inlets.

daughter ion is the ion, m/z 174, is 34% relative abundance. Figure 4-10 B presents the IRMPD spectrum of the protonated tylosin using a pulsed inlet of helium with 10 laser pulses. The reduced pressures obtained during laser firing increases the photodissociation efficiency (18% for constant inlet vs 92% for pulsed inlet), as evidenced by the decreased ion signal intensity for the parent ion at m/z 916 and the more intense signal obtained for the daughter ion at m/z 174. Several daughter ions that were not observed at the higher pressures during IRMPD are now detected, including the daughter ions at m/z 598, 408, 372, and 192.

Collision-Induced Dissociation Versus IRMPD of Tylosin

Figure 4-11 displays a comparison of the CID and IRMPD spectra for the $(M+H)^+$ ion of tylosin at m/z 916. Using a q_z of 0.15 (LMCO = 152), the CID spectrum of the $(M+H)^+$ ion of tylosin is shown in figure 4-11 A. The CID spectrum is highlighted by an intense daughter ion at m/z 772, and several low intensity ions at m/z 804, 756, 742, 598, 408, and 174. The IRMPD spectrum for the $(M+H)^+$ ion of tylosin ($q_z = 0.07$, LMCO = 71) using a pulsed inlet of helium and 10 laser pulses is presented in figure 4-11 B (same spectrum presented in figures 4-9 and 4-10 B). The IRMPD spectrum of tylosin is highlighted by an intense signal for the daughter ion at m/z 174. When compared with the CID spectrum, the same daughter ions are observed; several daughters ions not detected with CID are also observed, including the ions at m/z 192 and 156. The detection of more low m/z ions using IRMPD, but not with CID, was expected due to higher fragmentation efficiency (higher energy deposition).

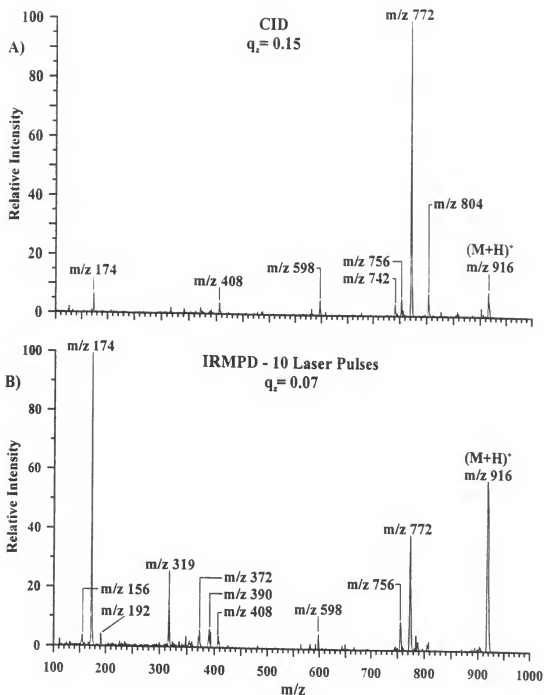


Figure 4-11. Comparison between CID and IRMPD of the $(M+H)^+$ ion of tylosin at m/z 916. (A) CID spectrum acquired at a pressure of 1×10^{-4} torr, $q_z = 0.15$, frequency = 56 kHz, and amplitude = 1.2 V. (B) IRMPD spectrum acquired using a dual pulsed inlet of helium at a $q_z = 0.07$.

IRMPD of Polymyxin B₁ Using a Pulsed-Inlet of Helium

Although the IRMPD technique has been particularly amenable to the analysis of carbohydrate antibiotics due to the absorption of IR energy by C-O-C linkage, the improved IRMPD efficiencies obtained with a pulsed introduction of helium is also beneficial for the analysis of peptide antibiotics. Figure 4-12 presents the IRMPD spectrum of the $(M+2H)^{+2}$ ion of polymyxin B₁ at m/z 602 using a pulsed inlet of helium and 13 laser pulses. The RF voltage was set to a q_z value of 0.37 (LMCO=250) for the m/z 602 ion. Polymyxin B₁ is a cyclic amphipathic peptide antibiotic that contains free amino acid groups and a fatty acid tail. Cleavage of the amide bond directly below the cyclic peptide ring produces the singly charged daughter ion at m/z 762. Loss of one water molecule gives the daughter ions at m/z 744. Cleavage of the second amide bond below the cyclic peptide produces the daughter ion at m/z 862, along with the dehydration fragment ion observed at m/z 844. Also observed is the daughter ion at m/z 963 due to cleavage of the terminal amide linkage. Successive losses of water from this ion give the daughter ions at m/z 945 and 927. Several doubly charged daughter ions are also observed in the IRMPD spectrum. Successive water losses from the doubly charged precursor ion at m/z 602 produce the $(M-H_2O+2H)^{+2}$ ion at m/z 593, along with the $(M-2H_2O+2H)^{+2}$ ion at m/z 584. The ion at m/z 552 is doubly charged which corresponds to a loss of 100 Da; loss of C₇H₁₆ with cleavage β to the terminal amide linkage. One problem that is particularly noticeable in the IRMPD spectrum of polymyxin B₁ is the poor mass resolution achieved with the pulsed inlet of helium. Improvements in the mass

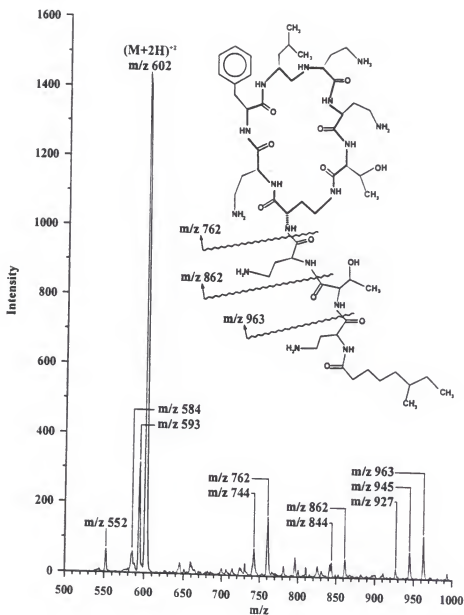


Figure 4-12. IRMPD spectrum of the $(M+2H)^{2+}$ ion of polymyxin B₁ using a dual pulsed inlet of helium with 13 laser pulses (LMCO=250).

resolution were achieved by decreasing the second pulse of helium before detection, but resulted in a loss of sensitivity for the daughter ions at m/z 762 and m/z 963. The photodissociation efficiency was lower for PMB_1 (approximately 20%) due to the lower photoabsorption cross section of the amide bond.

Conclusions

Infrared multiphoton photodissociation when combined with the quadrupole ion trap is an effective alternative to collision-induced dissociation for the structural elucidation of carbohydrate and peptide antibiotics. IRMPD allows the full characterization of many high molecular weight antibiotics, including daughter ions from the following reactions: dehydrations, glycosidic bond cleavages, glycan ring openings, and amide bond cleavages. One limitation to the implementation of IRMPD with the quadrupole ion trap is the helium buffer gas used both to increase the trapping efficiency of injected ions and to increase the detection efficiency of ions once trapped. Unfortunately, the typical 1 mtorr of helium (1×10^{-4} torr, uncorrected) continuously infused into the analyzer region for increased ion trap performance can collisionally deactivate photoexcited ions during the IRMPD process. However, by pulsing in helium buffer gas only during certain points of the scan function, reduced pressures (1×10^{-5} torr, uncorrected) are obtained in the analyzer region during laser irradiation. Several antibiotics were examined, including erythromycin, neomycin, and tylosin, using a two-pulsed inlet of helium; the results were significantly improved when compared with the IRMPD spectra obtained at higher pressures (photodissociation efficiencies increased from 5-20% to above 90%).

More structurally significant daughter ions were also obtained with the IRMPD technique using a dual pulsed inlet of helium than with resonant excitation collision-induced dissociation. Of particular note was the ability to use lower RF trapping voltages during the IRMPD process, enabling the successful trapping and detection of several low mass ions not detected with CID.

Despite the utility of the IRMPD technique when combined with the quadrupole ion trap, the cost of the laser and the complexity of the IRMPD experiments will limit its use to only research laboratories. Nevertheless, the photodissociation process provides complementary information to that of CID, and may prove invaluable in the structural elucidation of many classes of biomolecules, including carbohydrates and carbohydrate antibiotics.

CHAPTER 5
QUANTITATIVE DETERMINATION OF POLYMYXIN B₁ IN HUMAN
PLASMA WITH LC/MS UTILIZING ELECTROSPRAY IONIZATION
COMBINED WITH A QUADRUPOLE ION TRAP

Introduction

Polymyxins (PMBs) are cyclic amphipathic peptide antibiotics that contain free amino acid groups and a fatty acid tail. The polymyxins are produced by *Bacillus polymyxa* and related species, and have been known as effective antibiotics since the period between 1947 and 1950.¹²³ Polymyxins bind to the lipid A portion of endotoxins (lipopolysaccharide, LPS) of Gram-negative bacteria and inhibit cell wall growth¹²³. The interaction of polymyxin and lipid A involves ionic forces between amino groups in PMB and negatively charged phosphate and carboxyl groups in lipid A. Also, hydrophobic interactions occur between the respective acyl chains of PMB and lipid A. Several reviews have been published detailing the biological and clinical aspects of the polymyxins.^{125,126}

Commercial samples of polymyxin B are separable by countercurrent distribution into two components which have been named polymyxin B₁ and B₂.¹²⁷ Polymyxin B₁ is the major component of clinical grade polymyxin B; its structure is shown in figure 5-1. Further studies have shown that other polymyxin preparations are also mixtures; there are ten compounds involved altogether.¹²⁷⁻¹²⁹ These compounds differ only in the nature of the fatty acid component attached to the core decapeptide. Several different analytical

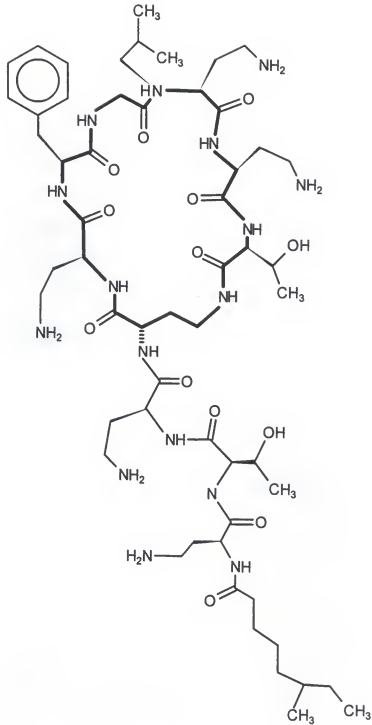


Figure 5-1. Structure of the cyclic decapeptide antibiotic polymyxin B1 showing free amino groups derived from α,γ -diaminobutyric acid and a fatty acid chain.

methods have been used to characterize the polymyxins, but the complete separation and identification of the various polymyxin components has only been obtained with high performance liquid chromatography (HPLC). The early research with polymyxins using liquid chromatography (LC) utilized UV-VIS absorbance spectrophotometry as the method of detection.¹³¹⁻¹³⁶ Unfortunately, the limited sensitivity achieved with UV-VIS detection does not allow the trace level examination of polymyxins in biological fluids.

Recently, electrospray ionization (ESI) mass spectrometry coupled with liquid chromatography has developed into the major analytical tool for the structural identification of small biomolecules in the pharmaceutical industry. In addition to qualitative studies, electrospray enables the quantitative determination of low levels of biomolecules due to the inherent sensitivity of the ESI/MS interface. When combined with HPLC, trace levels of drugs can be measured in relatively complex matrices such as plasma, serum, and urine^{8,136-140}.

Recently, interest has been shown in developing LC/MS and LC/MS/MS quantitative assays for polymyxin B₁ in human plasma. This chapter will first present the characterization of PMB₁ by electrospray (ESI)/ion trap mass spectrometry (ITMS). Next, the first attempts at developing an assay for PMB₁ in human plasma will be detailed. Finally, the first results at quantitating PMB₁ by LC/MS will be presented.

Experimental

Materials, Reagents, and Standards

HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific. ACS-grade formic acid, acetic acid, and trifluoroacetic acid (TFA) were also purchased from Fisher Scientific. Reserpine used as an internal standard was obtained from SIGMA

Chemical Co. The Sandoz Research Institute provided HPLC-purified polymyxin B₁ (sulphate salt). HPLC-grade water was obtained using a Millipore water purification system.

Human plasma was drawn from the author by the University of Florida Infirmary located on the UF campus. Blood was collected and centrifuged to obtain whole plasma used in all method development experiments. For the LC/MS method validation studies, human plasma was provided by the Sandoz Research Institute. Two anticoagulants were available: sodium heparin and K₃EDTA. Due to the possibility of the noncovalent binding of heparin with PMB₁, K₃EDTA was chosen as an anticoagulant in all plasma samples. Spec C18-AR solid phase extraction (SPE) cartridges with a 3 mL capacity (15 mg sorbent) containing a built-in filter were used in all sample cleanup procedures (obtained from Sandoz Research Institute).

Preparation of Standards

The reserpine internal standard solution (500 µg/mL) was prepared by dissolving 5 mg of reserpine in 10 mL of methanol. 100 µL of this solution was dissolved in 100 mL of water to prepare the reserpine stock solution at 500 ng/mL. The reserpine internal standard working solution (60 ng/mL) was prepared by diluting the 500 ng/mL stock solution by 1:8.33 with water. Plasma samples (500 µL) at each of seven different concentrations (plasma blanks with and without internal standard were also analyzed) were spiked with PMB₁ at Sandoz and delivered packed on dry ice. Care was taken to keep the plasma samples frozen until ready for analysis.

Pretreatment of Plasma Samples

Prior to electrospray LC/MS analysis, 100 μL of the working internal standard solution (60 ng/mL for day 1) was added to a 2 mL polypropylene tube. Next, 500 μL of human plasma (plasma blank or spiked with PMB₁) was pipetted into each tube and vortexed. After 3 minutes, 500 μL of 0.5% formic acid was added to each tube and vortexed. This solution was allowed to stand for 5 minutes. In order to precipitate any solid material or particulates, samples were centrifuged at 3000 rpm for 5 minutes. Each plasma sample was then ready for the SPE cleanup process.

One problem encountered during day 1 of the actual LC/MS analyses was the low level of reserpine observed on each chromatogram. Using a 100 μL reserpine spike with a concentration of 60 ng/mL produces an overall reserpine concentration of 10 ng/mL in 500 μL of plasma. This reserpine concentration is below the level for PMB₁ used in the actual analysis. Typically, internal standard levels are chosen in the midrange of the actual analyte concentrations (this would be in the range of 1-6000 ng/mL since PMB₁ concentrations range from 16.2-13254 ng/mL). A lower level of reserpine was chosen at first due to the high ionization efficiency obtained by collaborators at Sandoz with electrospray ionization on a triple quadrupole instrument (reserpine is commonly used in instrumental performance experiments for this reason). However, when integrating the lower signals of reserpine obtained, inconsistencies in peak integration developed. For this reason, on day 2 the reserpine internal standard working solution was increased from 60 ng/mL to 500 ng/mL (produced a reserpine concentration of 83.3 ng/mL in 500 μL of plasma). This resulted in larger peaks which were easier to integrate consistently.

SPE Plasma Sample Preparation for LC/MS Assay

Reserpine was chosen as an internal standard for two reasons: it contains two basic amine groups, and it produces a $(M+H)^+$ ion at m/z 609 (close to the m/z value of the doubly protonated adduct of PMB₁ at m/z 602). The plasma extraction procedure is outlined below:

- Pipette 100 μ L internal standard solution (reserpine at 60 ng/mL for day 1 and 500 ng/mL for day 2) into a 17x100 mm polypropylene tube, then add 500 μ L human plasma (containing different concentrations of PMB₁) into each tube and vortex. Add 500 μ L of 0.5% formic acid, vortex and allow it to stand for 5 min.
- Activate the SPEC C₁₈-AR cartridge (3 mL volume) with 0.5 mL of methanol followed by 0.5 mL 0.5% formic acid in water.
- Load the prepared plasma sample mixture onto the cartridge and allow to drain slowly.
- Wash the cartridge with 0.5 mL 0.5% formic acid followed by 0.5 mL 10% methanol/90% aqueous 0.5% formic acid.
- Dry the column for approximately 1 min (centrifuge at 3000 rpm).
- Elute PMB₁ from the cartridge with 0.5 mL of 95/5 methanol/0.5% formic acid .
- Place samples in a water bath at 45°C and dry the samples with a stream of nitrogen for 40 min.

- Reconstitute with 200 μL of 85/5/10 0.75% formic acid/methanol/acetonitrile, vortex, and inject 150 μL .

For the elution of PMB_1 from the SPE cartridges, different compositions of acetonitrile in both TFA and formic acid (0.5% to 1% aqueous solutions) were tried, and the final elution solvent mixture (95% methanol / 5% of 0.5% formic acid) was found to be optimal for the recovery of PMB_1 from human plasma.

High-Performance Liquid Chromatography/Electrospray Mass Spectrometry

Preliminary LC/MS and LC/MS/MS experiments were first conducted using an SSI Model 222C HPLC pump under isocratic conditions: 50/50 methanol water containing 0.05% trifluoroacetic acid (TFA). A Keystone Deltabond C_8 column (100 x 2.1 mm) with a Keystone C_8 guard column was used with isocratic conditions. For gradient conditions, an HP 1090 Series II liquid chromatograph was used containing a ternary mobile phase delivery system. A Waters Symmetry C_{18} (150 x 2.1 mm) with a YMC C_{18} guard column was used to obtain longer retention of PMB_1 on the stationary phase. The gradient consisted of 3 mobile phases: A) HPLC grade water with 0.75% formic acid, B) methanol with 0.75% formic acid, C) acetonitrile with 0.75% formic acid.

The gradient program used was:

<u>Time (min)</u>	<u>%A</u>	<u>%B</u>	<u>%C</u>
0.0	85	5	10
2.0	85	5	10
7.0	50	10	40
9.0	0	10	90
10	85	5	10

The flow rate was increased to 350 $\mu\text{L}/\text{min}$; the column temperature was increased to 40 $^{\circ}\text{C}$ using a Varian GC oven. A 150 μL sample loop was used with a Rheodyne 5100 manual injector.

The electrospray ionization/quadrupole ion trap mass spectrometer used in this part of the study was a Finnigan MAT LCQ. The LCQ consists of an ion source, ion optics, a quadrupole ion trap, and an ion detection system, as shown in figure 5-2. Although an atmospheric pressure chemical ionization source was available, the electrospray ionization source is better suited for compounds with acidic or basic functional groups such as the amine groups of polymyxin B₁. Typically the needle assembly is placed at 3-5 kV and is located 2-3 cm away from the heated stainless steel capillary. A nebulizing sheath flow of nitrogen gas assists in droplet formation. Charged droplets formed by the electrospray process are desolvated in the heated capillary (150-200 $^{\circ}\text{C}$), leading to ion formation. A tube lens placed at the exit aperture of the capillary gates ions into the octopole ion guide and also makes tube lens/CAD possible. Positive ions are stopped by placing approximately -140V on the tube lens.¹⁴² A skimmer at ground potential is located directly behind the tube lens assembly. To assist ions from the heated capillary to the ion trap region, two short octopole ion guides are used. Octopoles are efficient at transmitting ions through the higher pressures encountered in the first octopole region (1×10^{-3} torr).¹⁴³ The two octopoles are separated by an interoctopole lens, which serves three purposes. The small aperture of the interoctopole lens serves as a differential pumping barrier; a voltage applied to the lens (100V) transfers ions into octopole 2 while eliminating unwanted charged particles from passing and creating noise spikes at the

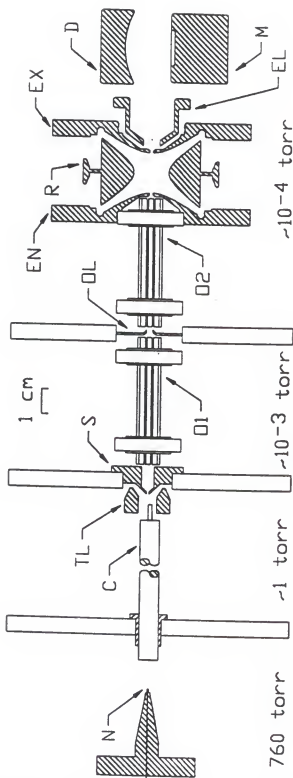


Figure 5-2. Schematic view of the Finnigan MAT LCQ showing electrospray needle assembly (N), heated capillary (C), tube lens (TL), skimmer (S), two octapoles (O1, O2), ion trap (EN, R, EX), exit lens (EL), and detector (D, M).

detector. Ions exiting the second octopole enter directly into the ion trap region. A 1 mtorr pressure of helium increases ion trapping efficiency. Also, an ion RF level that is optimum for the m/z (m/z range) of interest aids in trapping efficiency. The LCQ uses one to four different LMCO values (q_z) during ion injection in order to obtain optimal performance.¹⁴³ An exit lens held at +300 volts placed directly behind the exit endcap eliminates unwanted noise from the mass spectrum. A conversion dynode detector is located off axis.

MS and MS/MS experiments with PMB₁ were first performed using an ESI/ITMS instrument assembled at the University of Florida. PMB₁ solutions at a concentration of 50 ng/ μ L were continuously infused at a flow rate of 3 μ L/min using a 50/50 methanol/water mobile phase containing 1% acetic acid. MS and MS/MS data were obtained on the LCQ under the same conditions. ESI source conditions at low flow rates were sheath gas flow of nitrogen 30 arbitrary units, spray voltage 4.25 kV, capillary temperature 200 °C, capillary voltage 30V, and tube lens offset 30V.

For sensitivity studies on the LCQ, loop injections were performed without chromatography with a 5 μ L loop at a flow rate of 200 μ L/min using 50/50 methanol/water with 1% acetic acid. At high flow rates the ESI source conditions were sheath gas flow of nitrogen 80 arbitrary units, spray voltage 4.2 kV, capillary temperature 220 °C, capillary voltage 3V, and tube lens offset 30V.

Results And Discussion

Characterization of Polymyxin B₁ With Electrospray

The ESI/ITMS mass spectra of PMB₁ obtained at low flow rates using two different low mass cutoff values (LMCO) during ion injection are shown in Figure 5-3. A 1:1 methanol:water solution containing 1% acetic acid was used for preliminary experiments. At a LMCO value of 30, the triply protonated adduct of PMB₁ at m/z 402 dominates; the doubly protonated adduct at m/z 602 is also present. The ion at m/z 196 has not been identified. At a LMCO value of 60, the singly protonated adduct at m/z 1204 dominates; also observed is the doubly protonated adduct at m/z 602. As shown, varying the RF levels during ion injection allows the user control over m/z ranges that are efficiently trapped. A broader m/z range can be obtained by combining several different LMCO values into the same scan function, which is currently incorporated in the newer LCQ software. Further experiments using 0.05% TFA and 0.75% formic acid with methanol:water mixtures produced predominately the doubly protonated ion of PMB₁ at m/z 602.

In order to determine the fragmentation pathway to select for an LC/MS/MS assay, several CID experiments were conducted with PMB₁ in full scan acquisition mode. The MS/MS spectrum for the $(M+H)^+$ ion of PMB₁ at m/z 1204 obtained on the ESI/ITMS is shown in Figure 5-4. The ions at m/z 1186 and m/z 1168 arise from the consecutive losses of water. The daughter ion at m/z 1103 occurs due to cleavage of the terminal acyl chain β to the amide linkage. Cleavage of the distal amide linkage gives the ion at m/z 963; cleavage of the second amide bond gives the ion at m/z 762. The

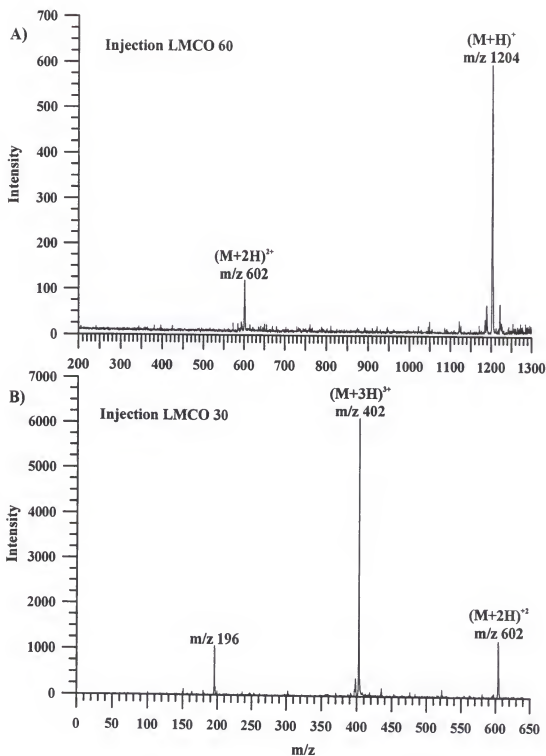


Figure 5-3. Electrospray mass spectra of polymyxin B₁ at two different LMCO values during ion injection. The $(M+H)^+$ and the $(M+2H)^{+2}$ ions are detailed at a LMCO of 60, and the $(M+3H)^{+3}$ adduct is the molecular ion at a LMCO of 30.

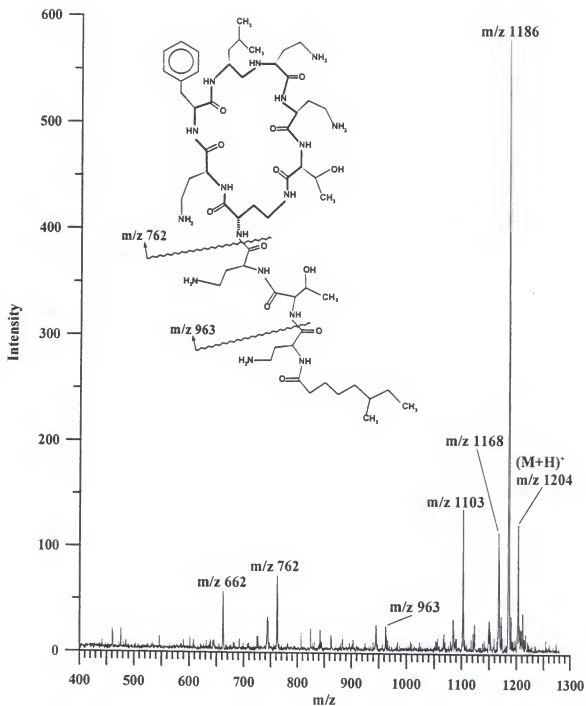


Figure 5-4. MS/MS spectrum of the protonated adduct of polymyxin B₁ at m/z 1204. Cleavage of the two amide bonds along the chain structure produced the two daughter ions at m/z 762 and m/z 963.

resonant excitation collision energy was set at 450 mV at a frequency of 116 kHz with the RF set at a LMCO of 400 ($q_z = 0.3$). Figure 5-5 shows the MS/MS spectrum for the $(M+2H)^{+2}$ ion of PMB_1 at m/z 602. Again, cleavage of both amide bonds gives the ions at m/z 963 and m/z 762. The doubly charged ions at m/z 593 and 584 are produced from the loss of one and two water molecules. The doubly charged ion at m/z 552 arises from cleavage of the acyl chain β to the amide linkage. The resonant excitation collision energy for the MS/MS spectrum was set to 100 mV at 116 kHz at a LMCO of 200 ($q_z = 0.3$).

Due to the many amine groups within the polymyxin structure, multiple protonation sites are available leading to doubly and triply charged polymyxin adducts (Figure 5-3). To examine the different MS/MS parameters required for complete fragmentation, a series of CID breakdown curves were generated using the Finnigan LCQ. Figure 5-6 presents the CID breakdown curves of the $(M+H)^{+}$ ion at m/z 1204, the $(M+2H)^{+2}$ ion at m/z 602, and the $(M+3H)^{+3}$ ion at m/z 402. The breakdown curves were generated to assess the required collision energy (measured as a percentage of the $5V_p$ which can be applied to the endcaps) necessary to obtain fragmentation information useful for an LC/MS/MS assay. Only structurally significant daughter ions were included in the plots, i.e. not daughter ions arising from losses of water.

For the singly protonated PMB_1 adduct at m/z 1204, fragmentation leads to the two structurally significant daughter ions at m/z 762 and m/z 963. The signal for these two ions starts to increase at a collision energy of 35%; the maximum signal intensity for the daughter ions is obtained at a collision energy of 45% (tickle voltage applied=2.3V).

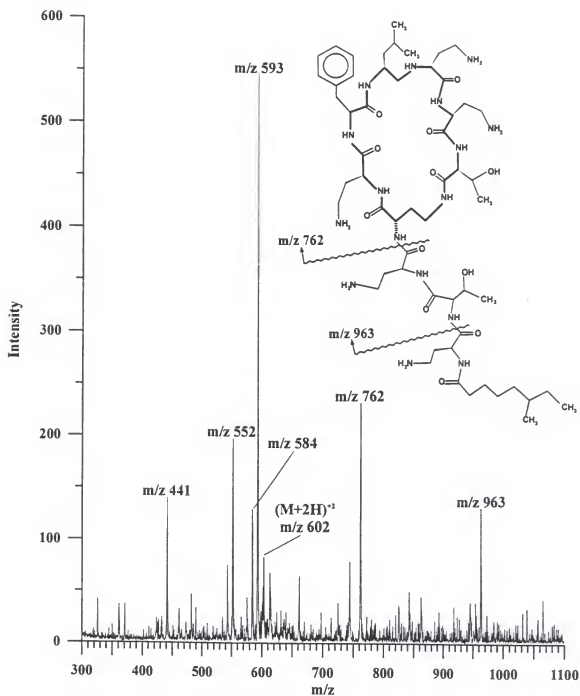


Figure 5-5. MS/MS spectrum of the $(M+2H)^{2+}$ ion of polymyxin B₁ at m/z 602.

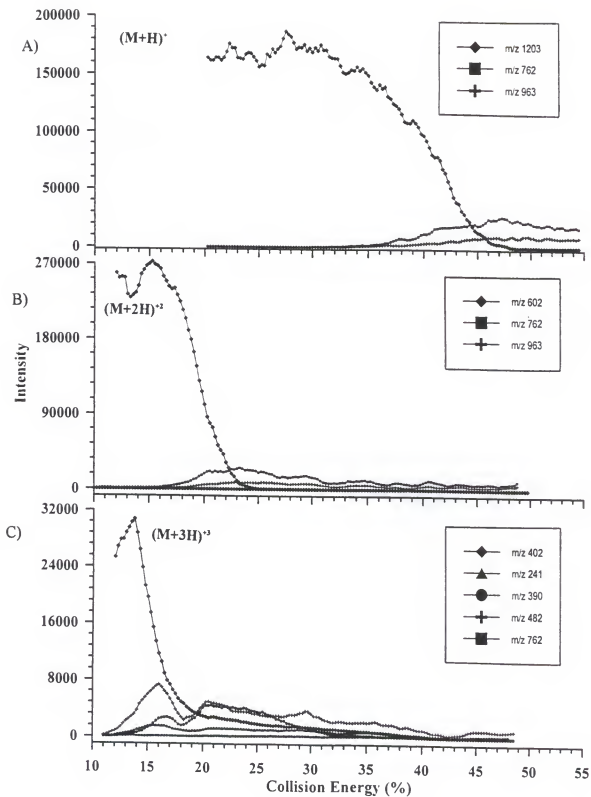


Figure 5-6. CID breakdown curves for polymyxin B₁: A) singly protonated adduct at m/z 1203, B) doubly protonated adduct at m/z 602, and C) triply protonated adduct at m/z 402.

For the doubly protonated adduct at m/z 602, fragmentation again produces the two characteristic singly charged ions at m/z 762 and m/z 963, starting at a collision energy of 17%; the maximum signal intensity for both ions occurs at a collision energy of 21%. The collision energy required to successfully fragment the doubly protonated adduct of PMB_1 is approximately half that required for the singly protonated adduct, as one might expect. This trend continues when varying the collision energy during the MS/MS scan for the triply protonated adduct. Here, no signal was obtained for the singly charged daughter ion at m/z 963, and a very low signal intensity for the singly charged fragment ion at m/z 762 (plotted in Figure 5-6 C). However, an intense signal was obtained for the doubly charged daughter ion at m/z 482 (doubly charged ion corresponding to the singly charged fragment at m/z 963) at a collision energy of 16%. Also observed were two triply charged daughter ions at m/z 241 and m/z 390. The daughter ion at m/z 390 arises from the loss of two water molecules to form the $(\text{M}+3\text{H}-2\text{H}_2\text{O})^{+3}$. The daughter ion at m/z 241 was identified as triply charged by examining the isotopic ratio using a high resolution scan on the ion trap mass spectrometer (C^{13} isotope was 0.33 m/z units higher), but no definite structural assignment has been made to date. Future experiments utilizing the MS^3 capabilities of the ion trap may aid in identifying the structure of this fragment ion. For the triply protonated adduct of PMB_1 , effective fragmentation occurs at collision energies above 18%, with a maximum signal intensity for the various fragment ions occurring over a wide energy range.

The trend towards reduced values of collision energies necessary to induce fragmentation of the parent PMB_1 adduct ions can easily be seen in figure 5-6 (A-C).

This trend can be easily explained since the kinetic energy imparted to an ion by a potential voltage is proportional to the number of charges on the ion. As a result, the lower collision energies are required for the fragmentation of multiply charged ions during the MS/MS experiment. Due to the large signal obtained at m/z 602 with a single stage of mass spectrometry, the fragmentation pathway from the doubly protonated parent ion at m/z 602 to the singly protonated daughter ion at m/z 762 was selected for further analysis in developing a reliable LC/MS/MS assay for PMB_1 in human plasma.

Method Development For Plasma Extraction

Before determining that solid-phase extraction (SPE) was best suited for the preparation of plasma samples for analysis of PMB_1 , several alternative extraction methods were examined. The simplest extraction method in concept is a liquid-liquid extraction, where ideally the organic solvent preferentially extracts the analyte from plasma without any interferences. Unfortunately, plasma is a complex matrix that includes a variety of compounds including various proteins and lipids. The analyte can bind with these proteins or lipids and thus not be efficiently extracted into the organic phase; the plasma proteins, lipids, and other components can also interfere by being extracted along with the analyte in the solvent extraction step. Several liquid-liquid extraction approaches for the analysis of PMB_1 were attempted, as detailed below, but all met with limited success.

First, several extraction solvents were used with a neutral plasma solution containing PMB_1 . PMB_1 is an amphipathic molecule, containing both a hydrophilic portion (ten amino acids forming a cyclic chain) and a hydrophobic lipid chain.

Therefore, the choice of appropriate solvent (polar versus nonpolar) was complicated. The solvents tried were diethyl ether, methylene chloride, ethyl acetate, chloroform, and hexane. Unfortunately, the best recovery was only 15%, obtained with ethyl acetate.

Since PMB₁ is a basic molecule (containing amine groups), several experiments were attempted using an alkaline plasma solution (sodium hydroxide added) at a pH in the range of 8-11. Again, the solvents used were diethyl ether, methylene chloride, ethyl acetate, chloroform, and hexane. The best recoveries were obtained at a pH of 9 using ethyl acetate, but the recovery of PMB₁ was still only 15%. A last series of experiments were run with an acidified plasma solution with zero recoveries. Unfortunately, a liquid-liquid extraction method for the recovery of PMB₁ in human plasma appeared unobtainable.

In order to simplify the plasma extraction scheme, several experiments were conducted using a protein-free plasma solution suitable for extraction with an organic solvent. The easiest method for obtaining a protein-free aqueous solution from plasma is to denature and precipitate the proteins and then isolate the filtrate. By denaturing the protein, the possibility of PMB₁ remaining bound to the protein is reduced, hopefully leaving the PMB₁ in the unbound form in solution (rather than in the precipitate). Several solvents and different reagents have been developed for protein precipitation, including ethanol, acetonitrile, and trichloroacetic acid (TCA).¹⁴¹ Ethanol and acetonitrile proved ineffectual at forming a protein precipitate. Trichloroacetic acid produced the best results, forming a large white precipitate on the addition of a 20% vol TCA/vol plasma solution (500 μ L of plasma required 100 μ L TCA). The plasma solution and precipitate

were next centrifuged and the solution decanted providing a clear aqueous solution. This solution was then used in several liquid-liquid extraction procedures. Unfortunately, recoveries were only 10% or less, giving the protein-free plasma sample no clear advantage for the analysis of PMB₁ using a liquid-liquid extraction method.

Over the last ten years, much attention has been focused on the use of solid phase extraction (SPE) cartridges for the analysis of drugs and metabolites in biological matrices (urine, blood, and plasma).¹⁴¹ An SPE cartridge is essentially an HPLC column reduced to a small scale. Ideally, the plasma containing the analyte is added to the SPE cartridge, washed (typically with water) to remove impurities such as proteins, and the analyte is then eluted from the cartridge with a efficient solvent, collected and analyzed. Another approach elutes the analyte of interest first without the elution of the interfering proteins from the plasma matrix. The first approach using SPE cartridges is more widely used.

Most of the HPLC work to date with polymyxins has been done with reverse phase columns. Therefore, a first attempt at developing a sample cleanup method utilized a C₁₈ SPE cartridge. The difficulty in SPE method development is finding the appropriate solvent for the wash and elution steps, and also determining the appropriate volume. Most SPE methods in the literature use a 100% aqueous wash step and a 100% organic solvent (typically methanol or acetonitrile) for elution. Using 100% methanol for the elution of PMB₁ resulted in clogging on the HPLC column during the analysis. This clogging, which led to an increase in column back pressure, was attributed to plasma proteins and/or lipids coming out of solution at the head of the column. To alleviate this

problem, an organic component was added to the wash step in hopes of removing the plasma impurities without eluting PMB₁. At this time 0.5% TFA was used during all wash and elution steps. Several different percentages of acetonitrile were tried during the wash step, including 2%, 5%, 10%, and 25%. Each fraction during the wash steps was collected and analyzed to insure PMB₁ was not eluting. The optimal wash step was obtained using a 500 μ L 0.5% TFA solution followed by a 500 μ L 10% acetonitrile solution containing 0.5% TFA. For the elution of PMB₁ after the wash step, several different percentages of acetonitrile were tried, including 30%, 50%, 75%, 95%, and 100% solutions containing 0.5% TFA. The optimal recovery for PMB₁ from plasma using a 500 μ L volume (limited due to small sorbent bed size in SPE cartridge) and without encountering clogging of the HPLC column was obtained with a 95% acetonitrile solution containing 0.5% TFA. The recoveries with this combination of solvents during the wash and elution steps varied from 60-68%. Through personal communication with Craig Abolin at the Sandoz Research Institute, it was learned that the use of trifluoroacetic acid with polymyxin B₁ can result in the formation of trifluoroacetate adducts and decreased LC/MS sensitivity for PMB₁. Formic acid was therefore chosen as a substitute organic modifier in the subsequent wash and elution steps; identical recoveries ranging from 60-67% were obtained.

LC/MS Results for the Recovery of Polymyxin B₁ in Human Plasma Calibration Curves for Day 1 and Day 2

The standard analytical curves for day 1 and 2 are illustrated in Figure 5-7. The calibration curves were generated using five replicate plasma samples (each spiked and

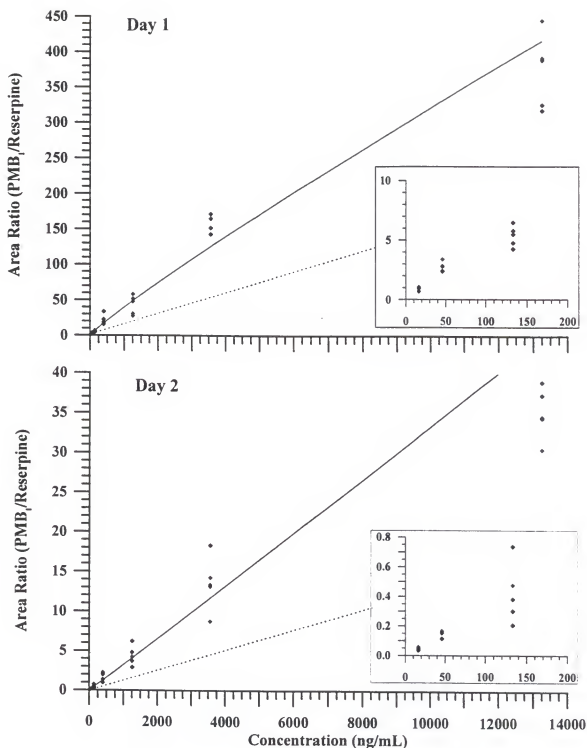


Figure 5-7. Calibration curves for the LC/MS assay for polymyxin B₁ in human plasma on two separate days. Linear best fit line is drawn on both curves. Insets are low concentration area ratios. Day 2 reserpine internal standard concentration is 8x higher than day 1.

extracted individually) at each of seven different concentrations covering three orders of magnitude (16-13,254 ng/mL). The high sensitivity of the electrospray/ion trap detection scheme, combined with the use of the internal standard reserpine, results in a dynamic range of 16-13,254 ng/mL. Detection limits ($S/N \geq 10:1$) were found to be 16 ng/mL (lower limits of detection were obtained with an MS/MS detection scheme). For day 1, a best fit nonlinear function for the line was obtained (Figure 5-7) giving a correlation coefficient (r^2) of 0.999 over the entire range. A linear fit was also obtained giving an r^2 value of 0.957. Four data points at the highest concentration level were all below the best fit linear line. A best fit linear line without these data points produced an improved fit with a correlation coefficient of 0.974. For day 2, using a different concentration of reserpine as an internal standard to improve peak area measurements, a best fit nonlinear function was obtained (figure 5-7) giving a correlation coefficient of 0.999. Again, the day 2 results at the highest concentration level were all below the best fit linear line (and also below the best fit line using a nonlinear function). This nonlinear behavior at the upper concentration range of the method analysis may have been due to overloading of the HPLC column. This column overloading, especially prevalent on day 2 where no guard column was used, led to broad, tailing peaks for PMB₁ which were difficult to assign peak area values during quantitation.

Recoveries of PMB₁ in Spiked Plasma

Recoveries of PMB₁ from the spiked human plasma were assigned by comparing the absolute peak areas after SPE extraction from plasma standards with those peak areas measured in the solvent (methanol/water) standards. A concentration of 1 ng/mL PMB₁

was spiked into plasma and compared with a standard at 1ng/mL. The relative recovery of PMB₁ from human plasma processed through C₁₈ SPE cartridges was consistently above 60%. Experiments on the recovery of reserpine were not conducted.

Analysis of Samples

Figure 5-8 details two typical mass chromatograms from the LC/MS assay for PMB₁ (doubly protonated adduct at m/z 602) in human plasma from day 2 containing 0 (A) and 45.7 ng/mL PMB₁ (B) with both plasma samples containing a 100 μ L spike at 500 ng/mL of reserpine as an internal standard (final concentration of reserpine in plasma is 83.3 ng/mL). Instead of setting up a scan function where only the two ions at m/z 602 and m/z 609 were analyzed (selected ion monitoring), a range of m/z values from m/z 600 to m/z 611 was analyzed (full scan acquisition). Using the chromatography conditions outlined in the experimental section, reserpine eluted at about 6.84 min in the plasma blank and 6.83 min in the plasma sample spiked with 45.7 ng/mL PMB₁. As shown in figure 5-8 B, polymyxin eluted at a retention time of 4.65 min. One reason for running plasma blanks is to insure there are no interfering compounds from the plasma matrix which coelute at the analyte retention time and produce ions at m/z 602. Five plasma blanks were also run on each day's analysis containing no reserpine, producing no interfering ions at m/z 609 coeluting at 6.83 min. There is a peak at m/z 602 occurring at 5.32 min (figure 5-8 A) and a peak in the TIC (total ion chromatogram) at 5.04 min (which the spectrum shows to be m/z 605); neither will affect the analysis of PMB₁ or reserpine. Spiked plasma samples were run in the order of low concentration (16.2 ng/mL) to high concentration (13254 ng/mL) on each day.

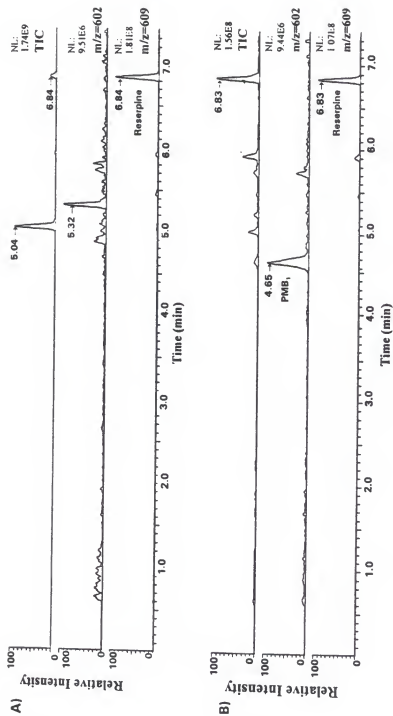


Figure 5-8. Mass chromatograms from the analysis of human plasma containing (A) zero and (B) 45.7 ng/mL polymyxin B₁ (m/z 602), each with 50 ng (100 μ L x 500 ng/mL) of internal standard reserpine (m/z 609).

LC/MS Assay Precision

The precision of an LC/MS assay describes the agreement between replicate measurements; the numerical value describing it should really be referred to as “imprecision”, so that a lower value will reflect lower imprecision (and thus greater precision).⁹⁹ The imprecision is typically measured by the standard deviation of the results in a set of replicate measurements; this is often expressed as the percent relative standard deviation (%RSD) or coefficient of variation (%CV). The within-day assay statistics for precision calculated for the quintuplicate analysis of each PMB₁ standard prepared in human plasma and analyzed on the same day are presented in Table 5-1. The percent relative standard deviation varied from 14% to 35% for day 1, and varied from 9.1% to 48% on day 2. However, overall, the LC/MS assay was imprecise; an average %RSD of 19% was calculated for day 1, and an average %RSD of 25% was calculated for day 2.

LC/MS Assay Accuracy

In determining the precision of the plasma samples prepared with differing concentrations of PMB₁, only the experimentally determined values were used in the calculation of imprecision. Accuracy, on the other hand, is the agreement between the best estimate of a quantity and its true value.⁹⁹ To determine the accuracy of the LC/MS assay for PMB₁ in human plasma, a statistical method called back-calculated accuracy was utilized. For this method the calibration standards were treated as unknowns and assigned concentration values based on the best fit standard curve parameters. The best fit parameters for day 1 were a nonlinear fit with the equation:

	Amount Spiked (ng/mL)	Area Ratio Average	Standard Deviation	%RSD (n=5)
	16.2	0.88	0.18	16
	45.7	2.75	0.40	15
	133	5.36	0.87	16
Day 1	393	21.3	7.37	35
	1255	43.1	13.4	31
	3554	154	13.0	8.4
	13254	373	52.1	14
	16.2	0.04	0.008	20
	45.7	0.14	0.02	13
	133	0.41	0.20	48
Day 2	393	1.74	0.55	32
	1255	4.37	1.24	28
	3554	13.5	3.41	25
	13254	35.0	3.22	9.1

Table 5-1. Within-day precision for the day 1 and day 2 results for the LC/MS assay for PMB₁ in human plasma. The internal standard for day 2 was 5x more concentrated, leading to lower area ratios.

$$Y = -0.0631 + 0.0792 X^{0.902} \quad (5-1)$$

where Y is the PMB₁/reserpine peak area ratio and X is the concentration of PMB₁. The best fit curve parameters for day 2 were obtained using a nonlinear function with the equation:

$$Y = -0.0277 + 0.00485 X^{0.956} \quad (5-2)$$

From these two equations the back-calculated accuracy for each plasma sample can be calculated using the following equation:

$$\%Accuracy_i = (1 - |x_i - \mu_i| / \mu_i) * 100\% \quad (5-3)$$

where x_i = the i^{th} observed concentration, and μ_i = the i^{th} expected concentration. A good model would have a high value for back-calculated accuracy and a low value for %CV. The results obtained for the back-calculated accuracy from day 1 and 2 are presented in Table 5-2. The %accuracy ranged from a low of 52.4% to a high of 99.7% on day 1, and ranged from 43.3% to 99.2% on day 2. One outlying value was eliminated from the day 1 results at a concentration of 393 ng/mL. The individual mean values from day 1 ranged from 73.9% to 86.3%. The overall mean for the back-calculated accuracy from day 1 was 81.3% with an overall %CV (coefficient of variation) of 16.6%. For day 2, the individual mean values ranged from 62.2% to 92.0%, a larger spread when compared to day 1. The overall mean value for day 2 was 78.0% with an overall %CV of 22.8%. These overall mean values for accuracy obtained on two different days were similar, although the different levels of the reserpine internal standard used on the two separate days prevent any thorough between-day statistical analysis. However, the

Day 1 Back-Calculated % Accuracy						
ng/mL	16.2	45.7	133	393	1255	3554
	75.1%	83.8%	88.9%	65.5%	96.8%	58.8%
	83.8%	99.7%	83.8%	-	93.9%	76.5%
	97.2%	97.1%	71.9%	90.9%	79.9%	84.7%
	83.4%	83.4%	99.0%	93.4%	52.4%	84.9%
	75.2%	75.2%	63.6%	91.9%	59.0%	64.7%
Individ. Mean	82.9%	83.8%	81.4%	85.4%	76.4%	73.9%
Individ. %CV	10.9%	20.6%	17.1%	15.6%	26.3%	16.0%
Overall Mean	81.3%					
Overall %CV	16.6%					
Day 2 Back-Calculated % Accuracy						
ng/mL	16.2	45.7	133	393	1255	3554
	99.2%	96.1%	77.8%	67.2%	83.3%	88.0%
	94.6%	97.3%	96.5%	59.4%	90.6%	44.1%
	89.9%	75.1%	50.5%	94.9%	65.4%	89.8%
	78.3%	95.4%	43.3%	43.6%	56.9%	72.0%
	88.0%	96.2%	62.0%	45.8%	95.1%	80.2%
Individ. Mean	90.0%	92.0%	66.0%	62.2%	78.3%	74.8%
Individ. %CV	8.7%	10.3%	32.5%	33.4%	21.0%	24.8%
Overall Mean	78.0%					
Overall %CV	22.8%					

Table 5-2. Within-day accuracy for the day 1 and day 2 results for the LC/MS assay for PMB₁ in human plasma.

overall accuracy of the LC/MS assay for PMB₁ in plasma (about 80%) can only be placed at adequate from the %accuracy values obtained for the day 1 and 2 results. Further experiments are necessary to obtain a more complete analysis of the accuracy of the assay. Until the precision of the analysis can be improved, it is not possible to obtain more accurate results.

Linearity of Polymyxin B₁ Peak Areas for LC/MS Assay

After a careful analysis of the peak area ratio calibration curves, the individual components of the LC/MS assay (PMB₁ and reserpine) were analyzed separately. Figure 5-9 presents the peak area data for PMB₁ plotted versus concentration of PMB₁ of each plasma sample. For day 1, using all 35 data points, the best linear fit line gives a correlation coefficient (r^2) of 0.937. Eliminating one outlying data point at the PMB₁ concentration of 13254 ng/mL improves the linearity, and gives an r^2 value of 0.978. Examining the PMB₁ peak area data from day 2, a correlation coefficient of 0.978 is also obtained. Again, eliminating one outlying data point at the high end concentration value of 13254 ng/mL improves the r^2 value to 0.991. As mentioned earlier, broad peak shapes at the high end concentrations were difficult to consistently assign accurate peak integration values. To assess the overall assay linearity, a comparison was conducted between the linearity of the area ratio curves of the LC/MS assay with the PMB₁ area curves using r^2 values for the best fit linear line. For day 1, an r^2 value of 0.957 was obtained for the area ratio calibration curve. For day 2, a correlation coefficient value of 0.964 was obtained. Using all 35 data points for day 1, both calibration curves (area ratio and PMB₁ area) give comparable correlation coefficients (0.957 and 0.937). No

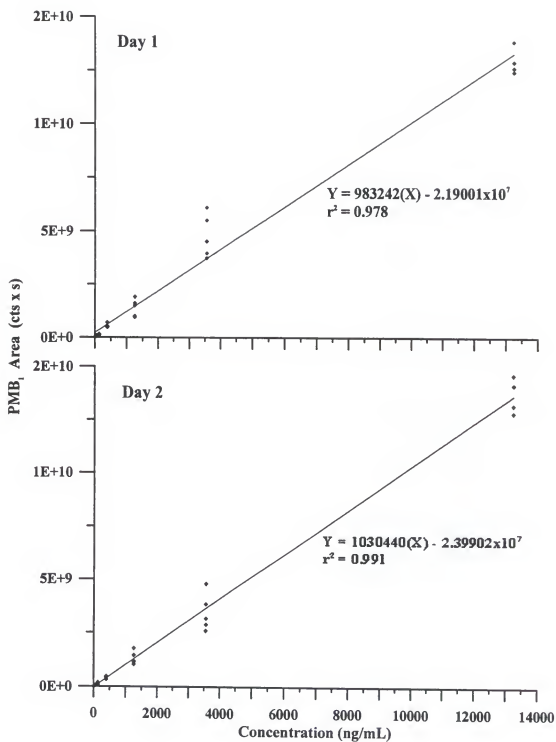


Figure 5-9. Polymyxin B₁ peak areas for day 1 and day 2 for the LC/MS assay

conclusions can be made, with the overall linearity of both curves being poor. However eliminating one data point from the PMB₁ area curve significantly improves the linearity to an r^2 value of 0.978. On day 2, the linearity of the PMB₁ area curve is consistently higher than the area ratio curve using both 34 and 35 data points. If the linearity of the PMB₁ area data is not the cause of the poor linearity from the area ratio curves, a problem may exist with the reserpine component of the LC/MS assay.

Reserpine Area Statistics

Figure 5-10 displays the reserpine area values for both day 1 and day 2. For day 2, a higher concentration of reserpine was used to improve the signal-to-noise ratio and expedite peak area integration. However, both plots display a large spread in reserpine peak area values across the PMB₁ plasma sample concentrations examined (the reserpine values should all fall in a narrow range and produce a straight line across the plot). Table 5-3 displays the precision statistics for the individual groups (N=7) of plasma sample plasma sample PMB₁ concentrations. The values for %RSD from day 1 ranged from 6.7% to 20.8% with an average %RSD = 16.7%. Only one group of PMB₁ concentrations (1255 ng/mL) examined consistently had %RSD values below 10%. For day 2, the range of %RSD values was 4.9% to 29.1% with an average %RSD value of 15.1%. Here, 3 values were below 10%, but the imprecision in the data collected at the PMB₁ concentrations of 393 ng/mL (%RSD = 29.1%) and 3554 ng/mL (%RSD = 24.8%) damage the overall statistics. The precision data in table 5-3 aid in the evaluation of the individual groupings, but to examine the overall precision of the reserpine peak area data every data point should be grouped together for the two separate days. Collecting the

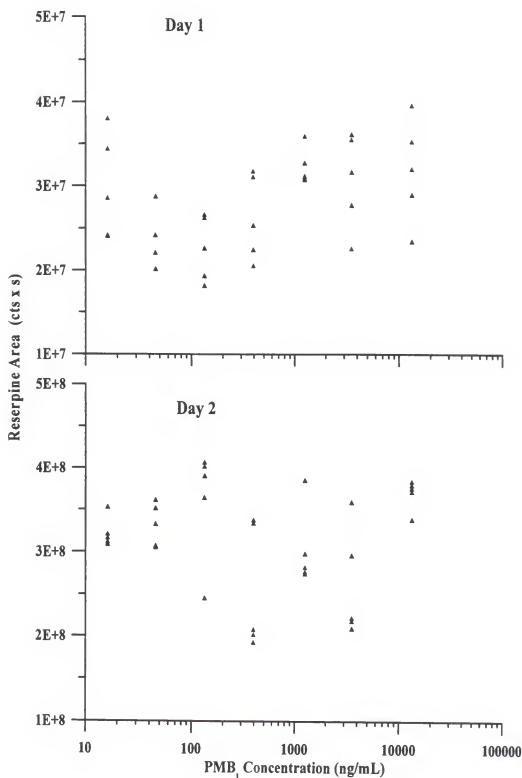


Figure 5-10. Reserpine (internal standard) peak area versus PMB₁ concentration for the LC/MS assay. Day 2 reserpine spike concentration increased to 500 ng/mL.

	Amount PMB1 Spiked (ng/mL)	Reserpine Area Average	Standard Deviation	%RSD (n=5)
	16.2	29872054	6211282	20.8
	45.7	23093600	3592376	15.6
	133	22623699	3872250	17.1
Day 1	393	26243076	5036208	19.2
	1255	32308676	2179380	6.7
	3554	30724407	5647503	18.4
	13254	31853021	6138889	19.3
	16.2	322289065	17647892	5.5
	45.7	331619972	25258856	7.6
	133	361704493	67113999	18.6
Day 2	393	254945638	74302119	29.1
	1255	303416927	46619768	15.4
	3554	260979192	64843001	24.8
	13254	369874120	18205221	4.9

Table 5-2. Reserpine peak area precision for day 1 and day 2.

peak area data from day 1 and calculating the average and standard deviation, an overall %RSD value of 20.8% is obtained. For day 2 an overall %RSD of 19.8% is calculated. On both days the total precision of the peak area values obtained was above an acceptable value for use as an internal standard (10%). Obviously, a different internal standard is required for an acceptable LC/MS assay, preferably a deuterated isotope of polymyxin B₁.

Conclusions

The combination of electrospray ionization/mass spectrometry with HPLC allows the quantitative determination of low levels of many analytes in complex biological matrices. The preliminary results of an LC/MS assay for the determination of polymyxin B₁ in human plasma have been presented in this chapter. Several different extraction schemes were attempted before determining that a solid-phase extraction procedure was best suited for optimal recoveries. Recoveries for the LC/MS assay for polymyxin B₁ in plasma were consistently above 60%. Due to the unavailability of deuterated polymyxin B₁, reserpine was chosen as an internal standard due to the basicity and its m/z value of 609 for its $(M+H)^+$ ion (close to the doubly protonated adduct of PMB₁ at m/z 602).

Spiked plasma samples were extracted using SPE cartridges and analyzed on two separate days. On day 2 the reserpine internal standard amount was increased to provide a more intense signal for peak integration. The limit of detection was found to be 16 ng/mL ($S/N \cong 10$) for the LC/MS assay; preliminary results using an MS/MS scan function have indicated lower limits of detection are feasible. The calibration curves generated for the two days were linear over the full concentration range, although a slight

nonlinear behavior at the highest concentration of 13,254 ng/mL was observed and attributed to column overloading. The assay precision results, however, were unacceptable, producing average %RSD of 19% for day 1, and 25% for day 2. The assay accuracy results were poor when including the entire 5 data points at each concentration. For day 1 an overall mean %accuracy of 81.3% was calculated, and a %accuracy of 78.0% was calculated for day 2.

To determine if the imprecision problems were primarily in the peak area measurements of reserpine or polymyxin B₁, separate plots were made for the individual components at each concentration. Examining the PMB₁ area data at each concentration for day 1 and day 2, linear plots were obtained, although with poor precision. The reserpine area for each PMB₁ concentration (same amount spiked at each concentration on each day) also showed poor precision; a %RSD of 20.8% was calculated for day 1, and a %RSD of 19.8% was calculated for day 2. This indicated that reserpine was a poor choice as an internal standard for the LC/MS assay. Future work using the d10-polymyxin B₁ will improve the results for an LC/M/MS assay for the determination of PMB₁ in human plasma.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

As illustrated by the IRMPD results for several high molecular weight antibiotics contained in this dissertation, the work begun three years has drawn to a conclusion. The IRMPD project analyzing antibiotics began with the completion in 1995 of a novel electrospray/quadrupole ion trap instrument built by Jim Stephenson for photodissociation studies^{5,6}. Using a pulsed CO₂ laser, four classes of biomolecules were previously characterized with the IRMPD process: peptides, oligonucleotides, oligosaccharides, and carbohydrate antibiotics^{5,6}. Due to the high photoabsorption cross section of the C-O-C linkage at 10.6 μm , carbohydrate antibiotics were chosen in this study for a more thorough characterization using the MS/MS capabilities of the IRMPD technique. Several questions were posed at the beginning of the project, including: Are there differences between CID and IRMPD spectra? Would the IRMPD technique open up dissociation channels not normally observed for CID of antibiotics? What is the effect of pressure on the IRMPD process? How may increased photodissociation efficiency be achieved with the IRMPD technique using the quadrupole ion trap? Is the selective cleavage of glycosidic bonds using IR radiation feasible? Would the IRMPD technique be useful in the analysis of high molecular weight non-covalent associations?

One problem encountered early on using the IRMPD technique with the quadrupole ion trap was the use of a 1 mtorr pressure (1×10^{-4} torr, uncorrected) of helium

buffer gas used to increase ion trap performance. Helium assists in the trapping of injected ions from the electrospray source, and also damps ion motion before detection, enhancing sensitivity and mass resolution. However, the use of a 1 mtorr pressure of helium buffer gas can collisionally deactivate photoexcited ions during the IRMPD process. Unfortunately, the first IRMPD experiments with antibiotics were conducted at relatively high analyzer manifold pressures ($9\text{--}11 \times 10^{-5}$ torr, uncorrected), and the photodissociation efficiencies obtained were poor. Several experiments were conducted at lower overall pressures, but poor sensitivity limited the results obtained.

Several experiments using the $(M+H)^+$ ion of the macrolide antibiotic erythromycin at m/z 734 indicated that an uncorrected pressure below 2×10^{-5} torr is necessary for photodissociation efficiencies of 80% or better. Obviously, a technique was needed to reduce or eliminate the adverse effects of the helium buffer gas during laser irradiation. To overcome the effects of collisional deactivation during IRMPD, a scan function was developed incorporating a dual pulsed introduction of helium. The first pulse of helium before ion injection effectively traps injected ions. A second pulse of helium before the mass-selective instability scan damps ion motion to the center of the ion trap, improving the detection efficiency of trapped ions. By utilizing this dual pulsed inlet of helium combined with the IRMPD process, enough time is allowed for removal of most of the helium before the laser irradiation step. Uncorrected manifold pressures of 1×10^{-5} torr are achieved during the IRMPD technique, significantly enhancing the photodissociation of trapped precursor ions.

IRMPD results using a pulsed inlet of helium were obtained for several antibiotics with both continuous wave and pulsed CO₂ lasers. Several interesting conclusions were drawn from the research. First, a pulsed inlet of helium is an effective method to improve the photodissociation efficiencies of the IRMPD process when combined with the quadrupole ion trap. Second, lower RF trapping voltages (q_z values) used during the photoactivation step of IRMPD allow the trapping and detection of lower m/z daughter ions. This might be particularly useful when elucidating the structure of a new antibiotic or an antibiotic that was modified slightly to improve its resistance effects. Direct comparisons of the IRMPD technique with collision-induced dissociation of the antibiotics erythromycin, tylosin and neomycin indicated that IRMPD provided more detailed structural information.

Third, the possibility of opening dissociation channels not typically observed with CID does exist with the IRMPD technique. IRMPD results with erythromycin indicated several daughter ions in the range of m/z 200 to m/z 500 that can only be explained by an opening of the core lactone ring (aglycone). Future IRMPD studies will focus on identifying these ring-opening fragments; other cyclic structures will also be analyzed to examine the possibility of ring-opening reactions with the IRMPD process. Finally, the well defined energy deposition of the photodissociation technique offers the ability to preferentially cleave the weaker non-covalent bonds (typically hydrogen bonds) of adducts formed during the gentle electrospray ionization process. Much attention has been focused on the analysis of non-covalent associations, especially for antibiotic-ligand interactions, but little attention has been directed towards alternative ion activation

techniques to assist in their analysis. IRMPD could prove useful in the detection of the individual components of an unknown non-covalent adduct, and may even prove useful in the measurement of binding constants.

Also included in this work were preliminary results from an LC/MS assay for the quantitative determination of polymyxin B₁ in human plasma. Using reserpine as an internal standard, plasma samples containing seven different concentrations of PMB₁ covering three orders of magnitude were analyzed on two separate days. Recoveries of PMB₁ using a solid-phase extraction technique were consistently above 60%. Calibration curves were linear, but precision results were poor. Accuracy results were also poor, with overall mean %accuracies below 85% calculated for the two separate days. Further examination of the methods used in the assay led to the determination that reserpine was a poor choice as an internal standard for the LC/MS assay for PMB₁. Preliminary experiments using an MS/MS procedure produced favorable results, and future work already underway will utilize the d-10 analogue of polymyxin B₁ as the internal standard for the LC/MS and LC/MS/MS assays for the determination of PMB₁ in human plasma.

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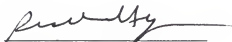
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BIOGRAPHICAL SKETCH

Stephen M. Boué was born in New Orleans, Louisiana, on September 29, 1962, to Gerald L. and Betty L. Boué. In 1980, he graduated from Rummel High School and went on to attend Louisiana State University. After two years, he returned home and attended the University of New Orleans. It was here that he was first introduced to mass spectrometry and electrospray in Dr. Richard Cole's laboratory. He also worked at Tulane's toxicology laboratory, where mass spectrometry was used for drug screening. He graduated with a B.S. in Chemistry in 1992.

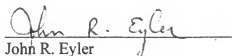
He decided to work with Dr. Richard Yost at the University of Florida. Here his interest in electrospray and ion traps led to his photodissociation research in collaboration with Dr. James Stephenson. After many hardships, he was successful in his endeavors. Upon graduation, Stephen will work with Dr. Richard Cole at the University of New Orleans.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Richard A. Yost, Chair
Professor of Chemistry

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John R. Eyler
Professor of Chemistry

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Willard W. Harrison
Professor of Chemistry

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David H. Powell
Associate Scientist of Chemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

A handwritten signature in dark ink, appearing to read "Joseph J. Delfino", written over a horizontal line.

Joseph J. Delfino
Professor of Environmental
Engineering Sciences

This dissertation was submitted to the Graduate Faculty of the Department of Chemistry in the College of Liberal Arts and Sciences and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1997

Dean, Graduate School